

**IMMUNOPATHOLOGICAL STUDIES IN THE OVINE LUNG DURING THE
COURSE OF NATURAL AND EXPERIMENTAL MAEDI-VISNA VIRUS
INFECTION**

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1994



DECLARATION

I hereby declare that the work presented in this thesis is my own work carried out entirely by myself except where specifically stated in the acknowledgements.

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February, 1994

ACKNOWLEDGEMENTS

I am specially grateful to Dr B. McGorum, Department of Veterinary Clinical Studies, R(D)SVS, for his great help in the writing of this thesis and to Dr L. Lujan, Department of Veterinary Pathology, Zaragoza, Spain, my collaborator in the work for Chapter 3 and a very good adviser and friend.

I am very thankful to Ms Lorna McLaren, Department of Veterinary Pathology, R(D)SVS for her excellent technical assistance in the bioassay for TGF- β and to Mrs L. Thornton, Department of Veterinary Clinical Studies, R(D)SVS, who introduced me into the world of tissue culture.

I much appreciate the considerable help of Mrs A. Baker (who performed the AGIDT test), Mr D.D.S. Collie, and Mr I. Pyrah, Department of Veterinary Pathology, R(D)SVS. They formed a wonderful team when dealing with the MVV-infected animals.

I am very appreciative to all the staff from the Marshall Building, especially to Mrs J. Docherty. They were irreplaceable in my work with the control sheep.

I am greatly indebted to Dr J. Hopkins, Department of Veterinary Pathology, R(D)SVS, for his many helpful discussions and for the supply of some reagents and to Mr A. Ross, Department of Veterinary Pathology, R(D)SVS for his skillful technical assistance and his endless patience.

I am most grateful to my supervisor Prof H.R.P. Miller for his guidance and all the time he dedicated to this thesis. I also thank Dr N.J. Watt, my other supervisor. I much appreciate the help of Dr G. Scott, Centre for Tropical Veterinary Medicine for statistical advice. I am very grateful to Mr N. McIntyre, Mr T. Ingman and Mrs L. Morris, Department of Veterinary Pathology, R(D)SVS, for their help with the cystopin preparations. I also thank to Dr R. Budjoso, Department of Veterinary Pathology, R(D)SVS, for kindly supplying the MAb IL-111A, Dr C. Burrells and G. Entrican, Moredun Institute, Edinburgh, Dr B. Dutia, Dr B. Blacklands, Ms D. Allem and Dr P. Bird, Department of Veterinary Pathology, R(D)SVS, Mr J. Jenkins and Dr C. Scudamore, Department of Veterinary Clinical Studies, R(D)SVS, Mr R. Munro for photography and Ms H. London for librarian services. Finally, I am indebted to the Spanish National Institute for Agricultural Research who sponsored me.

ABSTRACT OF THESIS

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Title of Thesis Immunopathological studies in the ovine lung during the course of
natural and experimental maedi-visna infection.
No. of words in the main text of Thesis

The pulmonary immunopathology of maedi-visna virus (MVV) infection was investigated, firstly performing *in vivo*, and *in vitro* studies and secondly, by reproducing the disease experimentally.

Peripheral blood (PB) and bronchoalveolar lavage fluid (BALF) cells of naturally MVV-infected and control sheep were phenotypically characterised by using single-colour flow cytometry. BALF from MVV-infected sheep had significantly increased percentages of lymphocytes, neutrophils and CD8⁺ lymphocytes, decreased percentages of CD4⁺ and CD5⁺ lymphocytes ($p < 0.05$), significant ($p < 0.001$) inversion of the CD4⁺/CD8⁺ ratio, increased MHC Class II expression on BALF macrophages and upregulated expression of LFA-1 and LFA-3 on BALF lymphocytes ($p < 0.05$). These data show the phenotypic changes in the cellular components of BALF during the course of natural MVV infection, and suggest a state of activation of BALF lymphocytes, and a pulmonary compartmentalisation of the cellular immune response during the course of the disease.

The stage of activation of BALF and PB lymphocytes in MVV-infected and control sheep was investigated by using dual-colour flow cytometry and several parameters of lymphocyte activation. Upregulation of DR and DQ MHC Class II molecules was present in BALF of MVV-infected animals, with no changes in cell size and complexity or increased expression of IL-2R, suggesting an impaired process of lymphocyte activation in BALF lymphocytes of these animals. There was no evidence of lymphocyte activation in PB.

To investigate if the impaired lymphocyte activation observed during the course of the natural disease was maintained under *in vitro* conditions, the response of BALF and PB lymphocytes to *in vitro* activation by exogenous mitogen was studied. BALF and PB lymphocytes from control and MVV-infected animals were cultured for three days after Con A stimulation, and IL-2R surface expression was quantified by dual-colour flow cytometry. Cultured BALF T lymphocytes from MVV-infected animals, after *in vitro* mitogen stimulation, expressed lower IL-2R than those of controls. These results suggest a dysfunction on cell-mediated immunity during maedi.

A model of experimental MVV infection was developed to study progressive early phenotypic changes in BALF T cells and to quantify TGF- β levels present in pulmonary epithelial lining fluid (PELF). Animals were inoculated by the respiratory route. Six animals received $10^{7.2}$ TCID₅₀ of MVV EV1 strain, two sheep were challenged with heat inactivated MVV EV1 strain and four sheep were sham-inoculated with virus-free buffer solution. Experimental infection was followed for 16 weeks post-inoculation (PI). Dual and single colour flow cytometry analyses and differential cell counts were performed on BALF cells. Precipitating antibodies to MVV developed in the MVV-inoculated animals between 2 and 4 weeks PI. Changes in the cellular composition of BALF were observed in all three groups of animals. Phenotypic changes in BALF T lymphocytes from MVV-inoculated animals were characterised by significantly ($p < 0.05$) decreased percentages of CD4⁺, CD5⁺ and $\gamma\delta$ T lymphocytes, a significantly ($p < 0.05$) increased percentage of CD8⁺ lymphocytes and a significant ($p < 0.05$) inversion of the CD4⁺/CD8⁺ ratio. Upregulated expression of MHC Class II and IL-2R molecules was observed on BALF T lymphocytes from all three groups of animals. No significant differences in the concentrations of plasma or PELF TGF- β were observed among the three groups of animals.

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ABBREVIATIONS

AGIDT	Agar gel immunodiffusion test
AIDS	Acquired immunodeficiency syndrome
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BCG	Bacille Calmette Guerin
BSA	Bovine serum albumin
CAE	Caprine arthritis-encephalitis
CD	Cluster of differentiation
Con A	Concanavalin A
CTL	Cytotoxic T lymphocytes
Da	Dalton
DAB	Diaminobenzidine
DCC	Differential cell count
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylene diaminetetraacetic acid
EIA	Equine infectious anemia
ELISA	Enzyme linked immunosorbent assay
ELF	Epithelial lining fluid
EV1	British isolate of MVV
Fab ₂	Antigen binding fraction of an antibody molecule
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FIV	Feline immunodeficiency virus

FL ₁	Green fluorescence
FL ₂	Red fluorescence
FSC	Forward angle light scatter
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HIC	Heat inactivated maedi-visna virus
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type 1
[³ H] TdR	Tritiated thymidine
HTLV	Human T cell leukemia virus
ICAM-1	Intercellular adhesion molecule-1
IFN- γ	Gamma interferon
Ig	Immunoglobulin
IL	Interleukin
IL-2R	Interleukin 2 receptor
ILD	Interstitial lung disease
IGF	Insulinlike growth factor
LCA	Leukocyte common antigen
LFA-1 and LFA-3	Lymphocyte function-associated antigens 1 and 3
LV-IFN	Lentivirus-induced interferon like factor
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MLC	Mixed lymphocyte culture
MLR	Mixed lymphocyte reaction
m.o.i.	Multiplicity of infection

MV	Maedi-visna
MVV	Maedi-visna virus
MW	Molecular weight
NCA	Neutrophil chemotactic activity
NK	Natural killer cells
NMS	Normal mouse serum
NRS	Normal rat serum
OLA	Ovine leukocyte antigens
OPP	Ovine progressive pneumonia
ORF	Open reading frames
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBMo	Peripheral blood monocytes
PBS	Phosphate buffered saline
PELF	Pulmonary epithelial lining fluid
PE-SA	Phycoerythrin-streptavidin complex
PHA	Phytohaemagglutinin
PGE ₂	Prostaglandin E ₂
PI	Post-inoculation
PMNs	Polymorphonuclear leukocytes
RNA	Ribonucleic acid
r.p.m.	Rotations per minute
SDS	Sodium dodecylsulphate
sIg	Surface immunoglobulin
SIV	Simian immunodeficiency virus
SPA	Sheep pulmonary adenomatosis
SSC	Side angle light scatter
TCID ₅₀ /cell	Tissue culture infective dose

TCR T cell receptor
TGF Transforming growth factor
 T_H T helper cells
TNF Tumor necrosis factor
 T_S T suppressor cells
UK United Kingdom
WBC White blood cells
w/v Weight for volume

GENERAL INTRODUCTION

Different aspects of the pulmonary immunopathology of maedi-visna virus (MVV) infection are presented by chapters. Each chapter begins with an outline of its aim and ends with an evaluation of the results.

Chapter 1 of this thesis is a literature review. Firstly, a review on MVV infection is presented: its history, causative agent, pathogenesis, clinical signs, lesions, epidemiology and control. Secondly, the pulmonary immune responses occurring during lentiviral infection are described using HIV as a model. A review of bronchoalveolar lavage (BAL) and flow cytometry techniques and a characterisation of sheep leukocyte molecules are also included in this chapter.

Chapter 2 is a description of the 'Materials and Methods' employed in the different chapters for the present work.

Chapter 3 studies the natural disease *in vivo*, concentrating on the cellular phenotypic changes that occur in bronchoalveolar lavage fluid (BALF) and peripheral blood (PB) during the course of MVV-infection.

Chapter 4 describes *in vivo* studies on the natural disease, investigating the stage of activation of BALF and PB lymphocytes in MVV-infected sheep.

Chapter 5 is an *in vitro* study of the response of BALF and PB lymphocytes to exogenous mitogen activation.

Chapter 6 studies experimental MVV infection, focussing attention on early phenotypic changes in BALF T cell subsets, and in the levels of the cytokine TGF- β in pulmonary epithelial lining fluid (PELF).

Finally, Chapter 7 discusses the main conclusions from the previous chapters in the light of the current understanding on ruminant lentiviruses, and the lentiviruses in general.

CHAPTER 1:INTRODUCTION

1.1. MAEDI-VISNA: A REVIEW

Maedi visna virus (MVV) is a member of the *Retroviridae* family. Representatives of this family are likely to infect many different animal species, causing a range of pathological processes (Varmus, 1988). All known retroviruses are classified according to their genomic structure into three main subfamilies: (1) *Spumaviridae*, which infect cats, nonhuman primates, and probably man without causing any apparent disease; (2) *Oncovirinae*, animal and human tumour viruses; (3) *Lentivirinae*, a group of nontransforming, cytopathic viruses that are known to be associated with states of immunodeficiency and neurologic disorders in several animal species.

With the discovery of a lentivirus as the causal agent of the human acquired immune deficiency syndrome (AIDS) there has been a resurgence of interest in the biology of these viruses and the pathogenesis of all the other animal diseases they cause namely maedi-visna (MV) of sheep, caprine-arthritis-encephalitis (CAE) (Crawford *et al*, 1980), equine infectious anaemia (EIA) (Dreguss and Lombard, 1954), feline immunodeficiency syndrome (Pedersen *et al*, 1987) and bovine immunodeficiency syndrome (Van der Maaten *et al*, 1972; Gonda *et al*, 1987).

Maedi-visna virus (MVV) is considered as the prototype of the animal lentiviruses, having recently acquired a great relevance from the comparative biological point of view. It is significant as a model of human retroviral diseases, sharing morphologic similarities and genome sequence homology with the aetiological agent of AIDS, (Sonigo *et al*, 1985). In addition, MVV causes a multisystemic disease of sheep which includes a diffuse interstitial pneumonia promising, hence, to be a useful prototype for the understanding of the human

interstitial lung diseases as well as for the study of the interaction between lentiviruses and their target cells, particularly macrophages.

1.1.1 APPROACH TO LENTIVIRUSES

Lentiviruses of the subfamily Lentivirinae are exogenous enveloped RNA viruses with a 60 to 70S single-stranded RNA genome and a RNA-dependent DNA polymerase (Haase and Varmus, 1973). With a complex biology and pathogenesis, they are unique and insidious pathogens, causing a chronic degenerative disease with involvement of different organ systems, variable prepatent period and clinical presentation and unpredictable recrudescence of the acute signs (Haase, 1986a).

They are particularly resistant against the host defence mechanisms (due to their ability to integrate their proviral DNA into host cell DNA, in particular the DNA of cells of the monocyte/macrophage lineage) and against the humoral immune defences (by undergoing antigenic variation). The 'Achilles heel' of these agents is their absolute dependence on body fluids for host-to-host transmission.

Replication of these viruses deserves a special mention as they are able to replicate even in the presence of virus specific immune responses. However, their replication *in vivo* is maintained at a restricted level throughout the infection, due to the virus-host interaction. Two main differences have been found between lentivirus and classical retrovirus replication (Narayan and Clements, 1990a);

1. Lentiviruses replicate efficiently in non-dividing end-stage cells, both in the host animal and in cell cultures, while other retroviruses require dividing cells for replication (Weiss *et al*, 1982).

2. Lentiviruses replicate their proviral DNA almost exclusively in the nucleus (Haase *et al*, 1982), while the synthesis of proviral DNA in oncogenic retroviruses takes place in the cytoplasm of the infected cell (Weiss *et al*, 1982).

Two types of clinical disease related to viral replication have been described (Narayan and Clements, 1989);

a) A *primary disease* caused directly by the Lentivirus and associated with the activation of virus replication in selective populations of macrophages. Primary lesions in this case would be due to the altered production of cytokines that are produced partly by the macrophages and partly by the lymphocytes as a response to antigen presentation by the immunologically activated, infected macrophages.

b) A *secondary disease* caused by opportunistic agents which proliferate as a result of the loss of function of activated helper T lymphocytes, which leads to a severe immunosuppression.

1.1.2 AN INTRODUCTION TO THE DISEASE

'Maedi' and 'visna', (Icelandic names, 'maedi' meaning *dyspnoea* and 'visna' meaning *wasting*) are considered as two histopathological and clinical forms of disease caused by MVV (Palsson, 1976; Dawson *et al*, 1979) which involve the lungs and the brain, respectively.

Morphological, biochemical and immunological studies of the causal agents of maedi and visna have proved that both nosological entities are caused by the same virus (Thormar, 1965; Thormar and Helgadóttir, 1965; Mehta and Thormar, 1975; Weiss *et al*, 1976; Stowring *et al*, 1979) and hence, the term MVV is now widely accepted to designate this virus (De Boer, 1975; Nathanson *et al*, 1976; Dawson, 1980; Martin and Stamp, 1980).

Progressive pneumonias of sheep resembling the Icelandic maedi/visna complex have been described under various names in different countries (Palsson, 1976), namely *Montana progressive pneumonia* or *ovine progressive pneumonia* (OPP) in the United States (Marsh, 1923), *Graaf Reinet* in South Africa (Mitchell, 1915; De Kock, 1929), *La Bohuite* in France (Lucam, 1942) and *Zwoegerziekte* in the Netherlands (Koens 1943; Ressang *et al*, 1968; De Boer, 1970). Histopathologically all these diseases are indistinguishable from maedi, and in the case of OPP and *Zwoegerziekte* they are virologically indiscernible. Although minor differences have been described in nucleic acid sequence and cross reaction of the internal protein of MVV and OPP viruses (Ressang *et al*, 1968; De Boer, 1970; Stone *et al*, 1971; Takemoto *et al*, 1971; Palsson, 1976; Weiss *et al*, 1976; Cutlip *et al*, 1979) they are closely similar in their physicochemical, morphological and immunological properties (De Boer, 1975; Cutlip *et al*, 1988).

1.1.3 HISTORICAL BACKGROUND

Loman (1862) first mentioned an ovine chronic respiratory disease in Dutch sheep from the island of Texel. In 1915 Mitchell described in South Africa a chronic ovine progressive pneumonia with similar characteristics to maedi. De Kock in 1929, reported as well in South Africa in the 'Graaf-Reinet' Experimental Station a disease very similar to the earlier one.

Marsh (1923) described in the United States another chronic progressive pneumonia, 'Montana disease', similar to that mentioned by De Kock, that he had observed in Montana and other north-western states since 1915. Some similarities between this pneumonia and the one described by Mitchell in 1915 were established later (Cowdry and Marsh, 1927).

Sigurdsson, Grimsson and Palsson first detected this nosologic entity in 1939 in Icelandic flocks and subsequently contributed much to the knowledge of MVV. The disease was introduced to Iceland by the importation of 20 Karakul rams from Halle, Germany in the summer of 1933 in an attempt to improve the productivity of Icelandic sheep and to promote production of Astrakhan coat (Gisslasson, 1947; 1966). Following a quarantine period of eight weeks, the imported sheep were distributed to 14 farms throughout the island (Perk, 1982). Epidemiological studies showed that two of the imported rams were carriers of MVV and one of them was also a carrier of sheep pulmonary adenomatosis (SPA). This latter animal died during the summer of 1934 showing symptoms of respiratory disease. The following winter SPA appeared on the farm where this ram had been kept, and on neighbouring farms and subsequently spread to many other farms causing heavy economic losses (Gisslasson, 1947). Maedi and SPA were commonly observed in the same flock and even in the same animal. Paradoxically, in the original flock of Karakul sheep no losses due to a disease resembling-maedi were detected (Palsson, 1976).

In 1937 an eradication program against SPA established by the Icelandic government managed to significantly reduce the incidence of this disease by slaughter in only two years. Only when the incidence of SPA diminished were two new conditions namely maedi and visna detected although, unfortunately, they had already extended to many flocks due to their insidious onset and to the concurrent infection with SPA. In 1941 the Icelandic government concentrated its eradication program on maedi and visna, managing to virtually eradicate visna in 1951 and maedi in 1954, although some sporadic outbreaks occurred until 1965 necessitating regular checking and control of the flocks until the middle seventies. Today Iceland is considered as a MVV-free country although this eradication policy proved expensive (Palsson, 1976; 1985; 1990).

MVV infection also occurs in Algeria (Achour *et al*, 1984), Belgium (Biront *et al*, 1981), Bulgaria (Pavloff, 1963), Canada (Bellavence *et al*, 1974), China (Deng *et al*, 1989), Denmark (Hoff-Jorgensen, 1971), France (Cottureau *et al*, 1977), Greece (Papadopoulos *et al*, 1971; Seimenis *et al*, 1985), Hungary (Suveges and Szeky, 1973), India (Rajya and Singh, 1964), Ireland (Guven, 1985), Israel (Nobel *et al*, 1973), Italy (Foglini *et al*, 1985), Kenya (Wandera, 1970), Mexico (Molina *et al*, 1986), Morocco (Mahin *et al*, 1984), Norway (Krogsrud and Udnes, 1978), Peru (Snyder *et al*, 1983), Sweden (Hugoson, 1978), Switzerland (Tontis, 1981), United Kingdom (Dawson *et al*, 1979) and USSR (Mitofanov and Yartsev, 1973).

1.1.4 THE VIRUS

MVV is a non oncogenic exogenous retrovirus belonging to the subfamily Lentivirinae ('lenti' is latin for 'slow') (Fenner, 1976), which exhibits a great tropism for the lymphoreticular and nervous system, where it replicates persistently but at a remarkably slow rate (Gudnadottir, 1974).

Despite the fact that MVV is morphologically similar to C-Type Oncornaviruses, antigenic and genetic criteria show them to be different (Haase, 1975). There is no evidence of oncogenic action of this lentivirus in the natural host (Petursson *et al*, 1979) although some tumoral transformations in murine cells have been described (Takemoto and Stone, 1971). As these transformations were not always observed (Brown and Thormar, 1975), it seems quite likely that those changes detected in the murine cells were due to spontaneous transformations of the infected cells or to the interaction of MVV with an endogenous oncovirus from the mouse (Teich, 1982).

1.1.4.1 Structure of MVV

The virus contains an enveloped, spherical, single stranded 60-70S RNA genome (Lin and Thormar, 1970a; Harter, 1976), characterised, as are all retroviruses,

by the presence of a RNA-dependent DNA polymerase (reverse transcriptase) which allows the virus to copy a DNA strand directly from the RNA genome in the virus particle (Bishop, 1978; Varmus, 1982; Stevenson and Bouffard, 1984). The viral nucleoid is closely juxtaposed to the outer membrane. The mature virion is approximately 80-120 nm in diameter. It contains a dense nucleoid (about 40 nm in diameter) and small spikes (about 8 nm long) project from the viral envelope. The virion matures by budding from the cytoplasmic membrane of the infected cells (Thormar, 1961; Filippi *et al*, 1982). Extracellular mature virions have a distinct intermediate layer and condensed electron-dense centrally located core with a characteristic shape, often forming a bar-shaped nucleoid (Coward *et al*, 1970; Dubois-Dalq *et al*, 1976).

1.1.4.2 Biochemical and biophysical properties

The virus contains approximately 60% proteins, 35% lipids, 3% carbohydrates and 2% RNA (Haase, 1975). Infective MVV particles sediment at a density of approximately 600S, similar to that of other retroviruses (Lin and Thormar, 1970a; Haase and Baringer, 1974). The virus can be maintained at -50°C and may withstand repeated freezing and thawing without appreciable loss of infectivity (Thormar, 1965; Sharma, 1982). MVV is inactivated by heat at 56°C for 10 min, diethyl alcohol for 20 h and by 0.04% formaldehyde, ethyl ether, chloroform, phenol, metaperiodate, oxidised spermine and trypsin (Thormar, 1965; Kennedy *et al*, 1968; Takemoto *et al*, 1971; Harter, 1976). The virus is stable in media with a pH between 5.1 and 10.

1.1.4.3 Substructure of MVV

a) Nucleic acid

A 60-70S single stranded RNA with a complex secondary structure and a molecular weight of $10-12 \times 10^6$ Da (Lin and Thormar, 1970a; Stone *et al*, 1971; Haase and Baringer, 1974; Brahic and Haase, 1981) constitutes 90-95% of the viral

genome, the rest being double-stranded DNA (Harter, 1976; Thormar, 1976). Two precursor units of 30-40S combine to form this nucleic acid (Brahic and Vigne, 1975), a process that takes place within the virion.

Heat denaturation divides the 70S RNA into two initial subunits of 35S. Apparently these subunits, whose molecular weight is 3.6×10^6 (Guillespie *et al*, 1973), have the same genetic information and hence the genome is often diploid and it could even be polyploid (Vigne *et al*, 1978). These subunits constitute the basic units for the performance of the reverse transcriptase in the viral replication (Haase and Baringer, 1974; Harter, 1976).

Three main regions containing the genes *env*, *gag* and *pol* have been differentiated in the viral genome, these genes coding respectively for the glycoproteins, the viral core proteins and the reverse transcriptase (Vigne *et al*, 1985). The genes are organised in the genome (5'-3') gag-pol-env according to a genetic structure similar to other retroviruses. Recent studies have shown a 9,202 nucleotide sequence (Sonigo *et al*, 1985), with a global composition as follows (Vigne *et al*, 1977); 21.4% uracil, 15.4% cytosine, 26.1% guanine and 37.1% adenine.

MVV genome presents seven 'open reading frames' (ORF), potential regions coding for proteins (Davis and Clements, 1989; Sargan and Bennet, 1989). These ORF are designated regions 'Q' and 'S' and their protein products could play a role in the regulation of the molecular events implied in the replication of the virus (Sonigo *et al*, 1985).

b) *Viral protein*

The exact number of proteins comprising the mature virion is not yet clear although between 10-25 of them have been identified (Haase and Baringer, 1974; Lin and Thormar, 1974). Only four of them: p25, p16, p14 and gp135 account for 90% of the total viral protein content, excluding the reverse transcriptase (Brahic and Haase, 1981). The main constituent of the spikes seems to be gp 135, a surface glycoprotein.

Antibodies against this glycoprotein have a neutralising activity and are type-specific (Narayan *et al*, 1981; Scott *et al*, 1984). The three major structural proteins p25 (p30), p16 and p14 have been called 'gag proteins' as they are encoded by this gene (Haase and Baringer, 1974; Lin and Thormar, 1979; Brahic and Haase, 1981; Perk, 1982; Vigne *et al*, 1985). p25 is the main viral core constituent representing about 40% of the total virion protein; p16 is related to the N-terminal extreme from the gag precursor and p14 seems to be a nucleic acid related protein (Sonigo *et al*, 1985).

c) *Reverse transcriptase (DNA-polymerase-RNA dependent)*

Reverse transcriptase is present in all retrovirus nuclei and is encoded through the gene 'pol' (Lin and Thormar, 1970b; Stone *et al*, 1971; Vigne *et al*, 1985). Two different forms of this enzyme have been described, with sedimentation coefficients of 6.9S and 6.3S and MW of 135,000 Da and 118,000 Da respectively (Sonigo *et al*, 1985).

1.1.4.4 MVV in cell cultures

MVV has been isolated by cultivation in cell cultures derived from ovine choroid plexus, lung and kidney (Gudnadottir and Palsson, 1967; Kennedy *et al*, 1968), testes (Takemoto *et al*, 1971), adrenals (Straub, 1970), ovine foetal lung, cornea and kidney (Thormar, 1976; Sharma, 1982), macrophages (Narayan *et al*, 1982) and cells derived from the synovial membranes from newborn kids (Narayan *et al*, 1980). Bovine, porcine and human cells can provide an optimum culture medium for MVV (Harter *et al*, 1968; MacIntyre *et al*, 1972), while cells from other species have failed to support viral production (Thormar and Sigurdadottir, 1962), except for Himalayan tahr ovary cells (Perk, 1988).

Viral attachment to cells in culture takes place after two hours of incubation at 37°C. Depending on the amount of initial inoculate the eclipse phase will oscillate

between 20-24 h (Petursson, 1990). During the next 15 h there will be an exponential increase, reaching a maximum titre 50 h post-infection (Harter and Choppin, 1967).

Infection of cell cultures with MVV is usually followed by a localised cytopathic effect within 24-36 h (Thormar, 1963; Sigurdadottir and Thormar, 1964; Harter and Choppin, 1967; Brahic and Haase, 1981; Caporale *et al*, 1985) consisting of cell fusion into giant syncytia, containing clusters of nuclei, formation of refractive mononucleated and giant stellar cells and, as the infection progresses, cytolysis. Due to all these changes there is a destruction of the cell culture in one or two weeks, with high titres of the virus in the culture supernatant (Thormar, 1963). The prototype Icelandic MV and OPP viruses are also cytolytic *in vitro* (Sigurdsson *et al*, 1960; Cutlip and Laird, 1976; Narayan *et al* 1977).

Ovine and caprine lentiviruses may also establish a prolonged productive infection in macrophages with minimal cytopathic effect (Klevjer-Anderson and McGuire, 1982; Narayan *et al*, 1982; Gendelman *et al*, 1984; Anderson *et al*, 1985) and furthermore, lentivirus-host cell interactions without apparent cytopathic effect have been described (Narayan *et al*, 1982).

1.1.4.5 *In vivo* cell tropism of MVV

The main targets of MVV infection *in vivo* are those cells from the monocyte-macrophage lineage (Narayan *et al*, 1982; Gendelman *et al*, 1985; Peluso *et al*, 1985). A selectivity in the macrophage populations which are infected by MVV is observed, with no reports of infection of liver Kupffer cells or histiocytes.

In vivo infection of peripheral blood dendritic cells has also been recently suggested (Gorrell *et al*, 1992). There is no substantiated evidence of MVV infection of lymphocytes (Carey and Dalziel, 1993).

1.1.4.6 Viral replication

The virus penetrates within the cell cytoplasm by fusing its envelope with the cell membrane (Harter, 1969; Brahic and Haase, 1981). Each virion contains two copies of viral RNA and both copies will be used by the 'reverse transcriptase' (Pyper *et al*, 1990). The transcription of the viral RNA to double stranded DNA starts within the cell cytoplasm and finishes within the nucleus (Haase and Varmus, 1973; Thormar, 1976; Brahic *et al*, 1977; Brahic and Haase, 1981). This DNA synthesis carries on during all the replication cycle giving rise to several copies per cell (Haase, 1986a), copies that will ensure the conservation of the viral genetic information for the production of the viral RNA and the viral progeny (Haase *et al*, 1982). A small fraction of this DNA is integrated within the cell genome (Clements *et al*, 1979), while a greater number of copies stay in the cell nucleus as 'free' DNA (Haase and Varmus, 1973; Haase *et al*, 1982). The synthesis of the viral RNA begins within the nucleus of the infected cell 5-7 h after infection and takes place in an exponential way until several thousands RNA copies per cell have been produced (Brahic *et al*, 1977). The exact mechanisms for the attachment of the different viral components are still unknown, but some complexes composed of RNA and several other polypeptides, that have been demonstrated to be infectious for ovine cells, have been detected (Filippi *et al*, 1982). These complexes would presumably give rise to viral particles by acquiring some material from the cytoplasmic cell membrane during the 'budding process' (Filippi *et al*, 1982).

1.1.4.7 Host range

Sheep and goats are the only species known to be susceptible to this disease (Banks *et al*, 1983). The disease in goats has been mainly reported in India (Rajya and Singh, 1964) although serological evidence of MVV infection in goats has also been recorded in Germany (Weinhold and Triemer, 1978) and in Norway (Krogsgrud and Udnes, 1978).

Several attempts to experimentally infect a number of laboratory and other domestic animals have failed (Palsson, 1976) and, although MVV can replicate in cultures of bovine or human cells, no evidence of *in vivo* infection in these species has been shown (Thormar, 1976).

1.1.5 PATHOGENESIS

MVV presents a complex biology and disease pathogenesis, as do all the viruses belonging to the Lentivirus subfamily. MV is a multi-systemic disease; following infection of the target cells there is a leukocyte-associated viremia (Gudnadottir, 1974; Nathanson *et al*, 1976) and the virus spreads to different organs including lungs, bronchial and mediastinal lymph nodes, spleen, kidneys, joints, salivary glands, mammary gland and choroid plexus (Gudnadottir and Palsson, 1967). The virus is also found in the milk of infected ewes (De Boer, 1970; Sihvonen, 1980). MVV infection provokes both an humoral and a cellular immune response, in spite of which the virus persists. The role of humoral and cellular immune responses in the development of MVV lesions is not known (Gudnadottir, 1974), but it has been reported that infection is not necessarily followed by the development of lesions (Sharma, 1982), and the typical MVV lesions characterised by mononuclear cell infiltration and lymphocytic hyperplasia may be immunologically mediated, as immunosuppression inhibits development of lesions in experimental visna (Nathanson *et al*, 1976; Panitch *et al*, 1976) while hyperimmunisation enhances the severity of the lesions (Nathanson *et al*, 1981).

As a compensation for their narrow host range and inefficient mechanism of transmission (depending almost exclusively on exchange of body fluids) the lentiviruses have elaborated complicated strategies for their constant replication in the immune competent host, the most remarkable being the integration of their genome into the host cell, which permits a latent infection without any expression of the viral genome even for years (Narayan and Clements, 1990a). Replication of lentiviruses *in*

vivo is maintained at a restricted level throughout the infection due to the virus-host interaction (Narayan and Clements, 1990a), and host susceptibility and virulence of the viral strain seem to play an important role in the severity of lesion development (Cheevers and McGuire, 1988).

Pathogenic mechanism of the lesions are not completely understood (Haase, 1986b). Viral persistence, the survival of the virus or latently infected cells even in the presence of antiviral immune responses, is fundamental to the pathogenesis of all lentiviral diseases, and in the case of MVV infection, accounts for the chronicity, progressive course and recrudescence of the disease (Cheevers and McGuire, 1988). Two basic mechanisms have been suggested to explain this feature;

1) *Antigenic drift or antigenic variation of neutralisation epitopes of virion surface glycoproteins.* Under the pressure of circulating antibodies some mutant strains can develop (Gudnadottir, 1974; Nathanson *et al*, 1976), which will not be neutralised by early sera (Narayan *et al*, 1987).

2) *Host restriction of viral gene expression.* This hypothesis states that the synthesis of viral antigen is restricted by suppression of viral gene expression and hence, virus infected cells are not eliminated by humoral or cell-mediated immune responses. Two mechanisms at least could contribute to this regulation, namely restricted expression of viral antigen by circulating monocytes and the generation of a soluble interferon-like factor (LV-IFN) by T cells during their interaction with MVV-infected macrophages (Narayan *et al*, 1985);

Restricted expression of the virus by circulating monocytes also plays a major role in the pathogenesis of MVV infection. Viral nucleic acid resides in a quiescent or proviral form in circulating monocytes (Narayan *et al*, 1982; Geballe *et al*, 1985; Gendelman *et al*, 1986). During monocyte maturation the virus is activated, leading to

a permissive infection (Narayan *et al*, 1982; Gendelman *et al*, 1986). Restricted viral antigen expression in the monocyte-macrophage series permits covert circulation of the virus throughout the body without inducing an immune response, a phenomenon that has been referred to as 'Trojan Horse' (Peluso *et al*, 1985), thus ensuring persistence and dissemination of the virus.

A final pathogenic mechanism is mediated by LV-IFN released from T lymphocytes during interaction with MVV-infected macrophages (Narayan *et al*, 1985). This LV-IFN not only delays maturation of monocytes to macrophages causing a reduction in virus production, but also induces the expression of MHC Class II antigens on the surface of local macrophages, so setting the stage for abnormal proliferation of lymphoid cells (Cheevers and McGuire, 1988), inducing production of PGE₂ by the macrophages (Narayan, 1988) and preventing virus-induced fusion (Zink *et al*, 1990).

1.1.6 MAEDI OR RESPIRATORY FORM

Maedi has been defined as a highly infectious, invariably fatal, insidious but continuously progressive interstitial pneumonia with a variable long incubation period (Sharma, 1982).

1.1.6.1 Clinical signs

Progressive emaciation despite a normal appetite, listlessness, physical weakness and exertional dyspnoea (with an increase in the respiratory rate up to 80-120/min) (Sigurdsson, 1954; Palsson, 1985; Brahic and Haase, 1981) are usually the earliest signs of the disease (Cutlip *et al*, 1988).

The main symptom in maedi is laboured breathing or 'dyspnoea' (Sigurdsson *et al*, 1952; Gonzalez, 1989) being barely perceptible at the beginning (only observed during exercise or physical stress), but as the disease progresses the dyspnoea increases and is present even at rest (De Boer *et al*, 1978; Dawson, 1980). At this

stage the animal displays dilated nostrils, flank breathing or 'pumping' (controlled by the accessory respiratory muscles), rhythmic jerk of the head (usually held high) and occasional mouth breathing. In the final stage of the disease, animals tend to lay on the floor first on sternal recumbency and finally in lateral recumbency until death occurs. Dry coughing and nasal discharge, although not constant features, could appear at any time during the course of the disease (Dawson, 1980).

Auscultation of the thorax, though seldom characteristic, can aid diagnosis, with vesicular breathing, crepitations and dry and moist rales being reported (Ressang *et al*, 1968).

A tendency to lag behind the flock and decreased milk production are also suggestive of maedi. Advanced cases may occasionally abort or produce small weak lambs (Dawson, 1980). Uncomplicated cases of maedi show a normal pulse rate and body temperature (Sigurdsson *et al*, 1952; Dawson, 1980; Palsson, 1985). Deaths (when no other complications supervene) occur in several months due to anoxia, although usually a secondary bacterial infection shortens the course of the disease in field conditions (Palsson, 1985). Though some animals may appear to recover temporarily, this entity is invariably fatal (Ressang *et al*, 1968; Dawson, 1980).

Stress factors such as pregnancy, bad weather, worm infestation, lactation, excessive physical exercise or shearing can precipitate the clinical phase of the disease (Sigurdsson, 1954; Palsson, 1976; Palsson, 1985; Cutlip *et al*, 1988) which, conversely, might be prolonged with good nursing (Dawson, 1980; Jones *et al*, 1982). However, according to Houwers and Van der Molen (1987), stress does not play such an important role in the onset of the disease. Slaughter of the diseased animals while still remaining in a moderately a good physical condition is a common practice.

1.1.6.2 Haematological findings

Sheep may show hypochromic anaemia, a significant increase in erythrocyte sedimentation rate, anisocytosis and poikilocytosis, persistent lymphocytosis,

individual variations in the haematologic results, slight increase of plasma fibrinogen, an increase in levels of serum immunoglobulins, significantly reduced average total protein in plasma (usually associated with hypoalbuminemia) and respiratory acidosis (Sigurdsson *et al*, 1952; Sigurdsson, 1954; Chauhan and Singh, 1970; Palsson, 1976; Molitor *et al*, 1979; Perk, 1982; Jones *et al*, 1982; Gonzalez, 1989).

1.1.6.3 Lesions in maedi

Macroscopic and microscopic lesions have been described by numerous authors (Sigurdsson and Palsson, 1958; Sigurdsson *et al*, 1962; Ressang *et al*, 1968; Georgsson and Palsson, 1971; Georgsson *et al*, 1976; Grimshaw *et al*, 1981; Van der Molen *et al*, 1985).

In maedi the gross pathological changes are confined to the thorax, except for those lesions arising secondary to emaciation. Histopathological lesions in maedi are not pathognomonic (Cutlip *et al*, 1978; De Boer *et al*, 1978), and because of that the diagnosis cannot be based solely on histopathological examination, both virological and/or serological methods being required. The histological changes in maedi are usually complicated by lesions caused by lungworms, and signs of terminal acute bronchopneumonia are often found.

Grossly, affected lungs are usually enlarged in size and are 2-4 times heavier than normal (from the normal range of 300-500 g to an average of 1200 g), remaining normal in shape (except for slightly rounder edges) but firm on palpation. On opening the thoracic cavity they collapse less than normal lungs. In uncomplicated cases rib impressions are evident on the surface of the caudal lobe. The consistency of affected lungs is diffusely rubbery and less elastic than usual, and occasionally adhesions may unite one lobe with another and connect the lung to the thoracic wall, although the general pattern is a smooth and shining pleural surface without fibrous adhesions

(Sheffield *et al*, 1980). The cut surface of affected lungs is dry. Although there is no sharp demarcation of lesions, diaphragmatic lobes may appear more severely affected.

Alteration of the lung colour is another consistent finding, usually being paler or greyish but ranging from gray-blue to gray-yellow or gray-brown. Occasionally, grey nodules may be observed. The presence of a subpleural grayish fine stippling homogeneously distributed through all the pulmonary parenchyma and constituted by small foci of 0.5-1.5 mm in diameter is also quite characteristic of maedi (Badiola, 1990).

In advanced cases, areas of red-grey consolidation (Badiola, 1990) may be observed, mainly in cranioventral areas, and on their cut surface a mucopurulent exudate often is present in the airways. These consolidated areas may be slightly elevated above the level of the surrounding tissue, associated with a diffuse fibrous pleuritis. When pulmonary oedema and congestion are extensive, areas of consolidation are difficult to detect.

Histopathologically, the interstitial pneumonia of maedi is characterised by:

1) Lymphoid tissue hyperplasia. This lymphoid proliferation was especially emphasised by Lucam (1942) in France who termed it 'lymphomatose pulmonaire maligne du mouton' describing it as being malignant. Today, it is accepted that the lymphoid proliferation is a reactive hyperplasia in response to the infective process, although is not pathognomonic of maedi or other viral pneumonias, as it seems to be a non specific response to various agents. The lymphoid tissue hyperplasia is the most consistent and prominent finding in maedi (Rajya and Singh, 1964; Gupta and Rajya, 1968; Sharma *et al*, 1974; Georgsson *et al*, 1976) and also one of the earliest histopathological signs (Sigurdsson *et al*, 1953). The lymphoid tissue hyperplasia is usually associated with formation of lymphoid follicles with active germinal centres in

peribronchiolar, perivascular or interstitial locations. Some foci of lymphocytes without arrangement into follicles may also be found.

Small greyish-yellow foci (seen on gross examination) are accumulations of proliferative lymphocytic cells with some of the lymphocytes infiltrating the interalveolar walls (Sigurdsson *et al*, 1952; Sigursson, 1954). In the early cases these foci are scattered but in the advanced cases they are extensive and prominent throughout the lungs.

2) Diffuse thickening of the interalveolar septae (Sigurdsson *et al*, 1952; Georgsson and Palsson, 1971). This is often quite pronounced, leading in some regions to a total obliteration of the alveoli and consolidation with practically no patent alveoli. This thickening of the interalveolar septae is mainly due to:

a) Mononuclear cell infiltration (Sigurdsson *et al*, 1952; Gisslasson, 1966) mainly with large mononuclear cells and to a lesser extent with lymphocytes, occasionally plasma cells and practically no polymorphonuclear leukocytes except in those areas of terminal acute bronchopneumonia.

b) Smooth muscle hyperplasia or hypertrophy with a diminished elasticity (Sigurdsson *et al*, 1952); there is a proliferation of smooth muscle fibres that may represent a compensation for the chronically diseased lung. It is usually more pronounced in bronchial and bronchiolar musculature, although the interalveolar septa may also be affected.

c) Existence of numerous histiocytes and fibrocytes and collagenous and reticular fibres, and in some lungs areas cuboidal epithelialisation of alveoli (Sigurdsson *et al*, 1952; Ressang *et al*, 1968; Georgsson and Palsson, 1971). All consolidated lung has atelectasis.

3) Hyperplasia of the epithelium in small bronchi and bronchioles. Due to this change the smaller bronchi display a hyperplastic appearance with a mucoid metaplasia and preponderance of goblet cells. Bronchial mucous glands may be widened and filled with mucus, and the bronchi also frequently contain mucus. This hyperplasia of epithelium in the terminal air passages is not specific to maedi as similar changes are observed in various viral pneumonias in man and in lambs infected experimentally with parainfluenza virus (Spencer, 1963).

This lesion is sometimes accompanied by disorganisation and epithelialisation of adjacent alveoli. Alveolar epithelialisation, also called 'foetalisation', consists of a partial transformation of the lining squamous epithelium of the alveoli into a cuboidal epithelium, and it takes place principally where the thickening and fibrosis of the interalveolar septa is most pronounced.

4) Vascular changes. Quite rare, they are only found in far advanced cases of maedi, being always of a moderate degree (Ressang *et al*, 1968; Georgsson and Palsson, 1971). They consist of an acute arteritis and peri-arteritis with a lymphocytic infiltration of the adventitia and media and endothelial proliferation. Hyalinisation of the media can occur, although it is exceptional. In addition, muscular hyperplasia and hypertrophy reduce the size of the lumen.

5) Intra-alveolar exudate (Georgsson and Palsson, 1971). In the absence of secondary bacterial infection there is seldom evidence of exudative or necrotising bronchitis or bronchiolitis, but in those cases in which such infection occurs, dense cellular exudates can be found in the alveoli. This intra-alveolar exudate consists mainly of desquamated macrophages, neutrophils and sporadic eosinophilic materials.

Alveolar macrophages may exhibit fusions, forming bi- and tri-nucleated cells, although the typical giant multinucleated cells which are present in MVV-infected tissue cultures are not found.

Regional lymph nodes (mediastinal and tracheobronchial) are also affected in maedi (Ressang *et al*, 1968; Georgsson and Palsson, 1971). Macroscopically, they appear swollen, markedly enlarged (from a normal weight of 10 to 15 g to an average of 40 g) and oedematous (slight to moderate oedema), without clear demarcation between cortical and medulla. This enlargement is mainly due to hyperplasia of the cortical lymphoid elements. Microscopically, lesions are characterised by a diffuse lymphoid hyperplasia. Some swelling and desquamation of the reticuloendothelial cells may be observed with accumulations of mononuclear cells in the medullary cords and of plasma cells and eosinophils in the sinuses. Neutrophils are rarely found.

1.1.7 VISNA OR NEUROLOGICAL FORM

Visna, the nervous form of the disease, was originally described by Sigurdsson and others (1957) as a slow, progressive disease of the central nervous system of sheep characterised by inflammation and demyelination. The first isolation of the causal virus took place in 1960 (Sigurdsson *et al*). Visna, as Cheevers and McGuire (1988) reported, was originally recognised as a complication of maedi in Icelandic sheep (Sigurdsson and Palsson, 1958) and occurs with low frequency in other breeds of sheep (Ressang *et al*, 1968; De Boer, 1975; Cutlip *et al*, 1979; Sheffield *et al*, 1980; Oliver *et al*, 1981a) and in other parts of the world (Foglini *et al*, 1985; Gonzalez *et al*, 1992). A possible explanation for the rare occurrence of visna in field cases of maedi could be the fact that virus-infected monocytes do not, under most circumstances, cross the blood-brain barrier or, if they do, they do not remain there (Narayan *et al*, 1984).

The incubation period is shorter than that for maedi (Dawson, 1980) and the clinical disease can be seen in animals over two years old. Experimentally, it can be transmitted by intracerebral inoculation of cell-free filtration of brain tissue and by intrapulmonary inoculation, although its mode of transmission in the field is still unknown (Gudnadottir and Palsson, 1965). The disease is invariably fatal, though during its clinical course some periods of relative normality or remission may occur. Its clinical course varies in length from a few weeks to several months or even a year.

1.1.8 OTHER CLINICAL FORMS OF MAEDI-VISNA

Chronic indurative mastitis

Although this clinical form of the disease has been recognised only recently, it is of great importance due to its high incidence being even more frequent than the respiratory form (Cheevers and McGuire, 1988; Lujan *et al*, 1991).

Arthritis

A chronic non suppurative arthritis, usually bilateral, has been described in carpal and tarsal joints of animals affected with MVV in USA. Although rare in presentation, it closely resembles that affecting the joints of goats with caprine arthritis-encephalitis (CAE) (Cutlip *et al*, 1985a; Narayan and Cork, 1985). Although naturally it occurs in animals over two years, experimentally it can appear within one year.

Vasculitis

Degenerative changes in blood vessels of MVV-infected animals have been reported (Cutlip *et al*, 1985b) but are always closely related to other lesions induced by the virus.

Testicular lesions

Although few data have been published on changes produced by viruses in genital infections (Watt, 1972), the testicular lesions found in rams infected by MVV suggest that the virus can be transmitted with semen (Palfi *et al*, 1989), in addition to respiratory secretions and saliva and so, infected rams probably play a role in transmitting infection (Suveges and Szeky, 1973).

Lesions in other organs

Spleen: An acute splenitis has been described (Sheffield *et al*, 1980) where lymphoid follicles were consistently large and had prominent germinal centers.

Liver: Sigurdsson and others (1952), reported inflammatory changes consisting of small scattered infiltrates of lymphocytes and plasma cells without apparent connection with the vessels of the liver.

Kidneys: There may be infiltration of large proliferating lymphocytes at the cortico-medullary junction, spreading both into the medulla and cortex (Georgsson *et al*, 1976).

Heart: A generalised acute endocarditis, focal myocardial degeneration and slight hypertrophy of the right side of the heart have been found (Ressang *et al*, 1968).

1.1.9 EPIDEMIOLOGY AND CONTROL

1.1.9.1 Geographic distribution

Geographically, maedi has been detected in most major sheep producing areas of the world except for Australia and New Zealand (Jensen and Swift, 1988). Very high prevalence rates are observed in the western range flocks of the United States

where 50% of the total sheep population may be infected (Cutlip *et al*, 1977; Gates *et al*, 1978; Huffman *et al*, 1981).

1.1.9.2 Maedi/Visna in Great Britain

Although Jones and others (1982) first reported the clinical disease in the United Kingdom (UK), serological evidence of infection was first detected by Dawson and others (1979) in a flock intending to export rams.

It is thought that the importation of exotic breeds of sheep during the 1970s could have introduced the disease into the country as the first clinical cases were imported sheep, their progeny and those animals reared in contact with them. Today, however, the disease is spreading among commercial sheep flocks which have no connection with imported breeds (Pritchard *et al*, 1984; Pritchard and Spence, 1984; Pritchard and Dawson, 1987).

Maedi and SPA have been reported to co-exist in British flocks (Pritchard and Done, 1990; Watt *et al*, 1990; 1992a; Gunning and Shaw, 1993). Visna has also been reported in the UK (Pritchard and Done, 1990; Watt *et al*, 1990).

1.1.9.3 Economical losses

MVV infection causes heavy economic losses to the farmers (Sigurdsson *et al*, 1952), not only because of mortality and unthriftiness during the long course of the disease but also due to reproductive inefficiency and significantly reduced longevity (Blood and Henderson, 1983; Houwers, 1990).

The reports of subclinical MVV infections causing indurative mastitis (Van der Molen *et al*, 1985) and other deleterious effects on productivity (Dohoo *et al*, 1987) must also be of concern to the sheep industry.

1.1.9.4 Susceptibility to MVV

1.1.9.4.1 Breed susceptibility

Different authors have different points of view concerning this subject:

Cutlip and others (1988) and Jensen and Swift (1988) indicated that all breeds of sheep are susceptible to MVV infection, although some breeds are more resistant than others to the development of the disease after the infection (Cutlip *et al*, 1986).

Palsson (1976) considered genetic predisposition to be the most important factor and hence, certain strains of sheep within the same breed will be more resistant to clinical disease than others.

Finally, Gates and others (1978) and Narayan and Clements (1989) believe in breed susceptibility, reporting that Border Leicester sheep in the USA and Texel sheep in Holland are more susceptible to infection. Serological studies on field samples in USA have also shown a higher prevalence of infection in some breeds than in others (Gates *et al*, 1978; Light *et al*, 1979).

1.1.9.4.2 Age susceptibility

Although the age-specific prevalence shows that the proportion of infected animals increases with age (Jensen and Swift, 1988), which could indicate either lateral transmission of the virus or delayed seroconversion after infection at birth, some other authors have reported that natural disease may occur at any age in adult sheep (Cross *et al*, 1975). Though more common in 2-3 year old sheep, disease has been described in lambs from infected sheep (Cross *et al*, 1975) and pulmonary lesions have been described in foetuses after the intrauterine inoculation of the virus (Cutlip *et al*, 1982) and in young lambs after intratracheal inoculation of low-passage virus (Lairmore *et al*, 1986).

1.1.9.5 Concurrent infection with MVV and Sheep Pulmonary Adenomatosis

MVV spreads more quickly and produces more severe losses in those flocks that are affected with SPA (Houwens and Terpstra, 1984; Dawson *et al*, 1985; 1990). The possible explanations for this synergism between infection with SPA and MVV are: firstly, the increased numbers of alveolar macrophages present in SPA lungs, particularly adjacent to the tumour tissue (Hunter and Munro, 1983) may provide optimal conditions for effective MVV replication; secondly, typical SPA bronchial secretions and subsequent coughing may increase the number of airborne MVV droplets; and thirdly, MVV seems able to integrate into the tumour cells' genome, helping the simultaneous transmission of both viruses from infected to healthy animals.

In advanced mixed infections it can be difficult to distinguish between the lesions due to MVV and those due to SPA.

1.1.9.6 Transmission

The virus is transmitted primarily from dam to lamb through the *colostrum and milk* (De Boer, 1970; Palsson, 1976; De Boer *et al*, 1979; Dawson, 1987; Narayan and Clements, 1989) and isolation of MVV from milk has been reported up to 5 months after lambing (De Boer, 1970). The proviral state ensures that the virus remains closely cell-associated, thus the transference of infected cells between animals is necessary for transmission. Differentiated macrophages from the mammary gland, present both in colostrum and milk are the target cell for viral replication (Narayan and Clements, 1989) and because of this, any concurrent infection leading to an increase in the extrusion of macrophages into the milk (such as mastitis) will increase the load of lentivirus in the milk (Kennedy-Stoskopf *et al*, 1985a). Infected macrophages probably pass through the intestinal epithelium of the neonate to establish infection.

The longer a dam has been infected, the sooner infection can be detected in her lambs after birth, as lambs will be exposed to higher numbers of virus (Sihvonen, 1981).

Horizontal or contact transmission. Droplet infection via the respiratory route was the most likely method of transmission during the epidemics of the disease in Iceland (Palsson, 1976). However, under experimental conditions it has been difficult to prove respiratory transmission, and very close contact between affected and unaffected animals seem to be necessary (Sigurdsson, 1954; Gisslasson, 1966; Blood and Henderson, 1983) although infection can also become well established among traditionally managed unhoused sheep (Pritchard *et al*, 1984; Houwers and Van der Molen, 1987). Overcrowding and close housing of the animal for a long period facilitates the spread of the infection (Sharma *et al*, 1975). Some authors, however, consider that respiratory transmission is less important than milk transmission (Sigurdsson *et al*, 1953).

Saliva has been reported to be another possible source of virus (Straub, 1970; Narayan and Cork, 1985).

Although epizootiological data implicate *rams as sources of infection* (Suveges and Szeky, 1973), no data has been published on the mode of spread (Palfi *et al*, 1989). It is believed that two rams were implicated in introducing the disease in Iceland (Palsson, 1976) and in Norway (Krogsgard and Udnes, 1978). However, experiments to date have failed to demonstrate that semen from MVV infective rams is infective (Dawson, 1987).

Blood does not seem to be particularly infective in spite of the persistent viraemia during MVV infection (Dawson, 1987). The low infective capacity of blood could be due to the fact that the virus is only present in one in 10^6 to 10^7 leukocytes and only as dormant proviral DNA (Peluso *et al*, 1985).

No evidence has been found to suggest that sheep lungworm *Muellerius capillaris* (De Boer *et al*, 1979) or the sheep ked *Melophagus ovinus* (Sigurdsson *et al*, 1953) were implicated in the transmission of MVV infection.

Indirect transmission is probably uncommon (Dawson, 1980, 1987; Straub, 1985; Palate and Koenen, 1987; Sharp, 1988).

Experimental studies have demonstrated transmission via *drinking water contaminated with faeces from infected sheep* (Sigurdsson *et al*, 1953). Nevertheless, the infectivity of faeces and urine has not been examined thoroughly enough to allow generalisation (Gudnadottir, 1974).

Opinions regarding the importance of *vertical transmission* differ. Cross and others (1975) and Cutlip and others (1981) believe that transmission *in utero*, although rare, can occur either through the uterine wall or via the germ cell. Hoff-Jorgensen (1977) isolated MVV from three lambs delivered close to term from infected dams by caesarian section. However, the epidemiological study by De Boer and others (1979) failed to demonstrate evidence for transplacental infection and transplacental transmission could not be demonstrated in the experiments of Sihvonen (1980).

Cross and others (1975) described interstitial pneumonia in four months old lambs which were hysterectomy-derived from sheep suffering from maedi, but some other authors (De Boer *et al*, 1979), consider that those observed changes in lungs are not conclusive for vertical transmission, and claim that the diagnosis was not substantiated by serological investigation and virus isolation. Furthermore, the virus could have been transmitted into the foetuses during the surgical manipulation.

The difficulty in isolating virus from the foetuses taken by caesarian section from MVV-infected sheep could be explained by considering the particular mode of replication of lentivirus rather than a lack of foetal susceptibility. Hence, virus might exist in the foetuses as a provirus (in a non-infectious, non recognisable state) and only under certain conditions, or after the animals have reached a certain age, would

infectious viral particles be produced. The presence of the provirus could be shown by using a hybridisation technique.

1.1.9.7 Control

There is no effective treatment for this disease, nor is there any available vaccine (Cutlip and Lehmkuhl, 1986). Vaccines would be expensive and not too efficient (Narayan and Clements, 1989) due to the antigenic variation of the virus (Stevenson and Bouffard, 1984). Moreover, vaccinated sheep, although producing complement-fixing antibodies, are not protected (Haase, 1986b). Hence, vaccination at the outset of infection might reduce the number of potential cells involved in the pathological changes, but will not prevent lentivirus infection (Haase, 1986b). Experimentally, however, inhibition of viral DNA synthesis (Haase and Levinson, 1973), intracellular action with interferon (Carroll *et al*, 1978; Narayan *et al*, 1985), control of the transcription and translation of the lentiviruses genome (Haase, 1986b) and the development of a defective virus vector which would stabilise the dormant state of infection (Staskus, unpublished) are the main points of interest for therapy, although still without practical applications. Treatment must be symptomatic; antiinflammatory drugs can be used to reduce joint pain and antibiotics to prevent secondary infections of the lungs.

The addition of clinically normal infected animals into a flock is the major route for the introduction of the disease. Even the negative serological testing of such animals does not guarantee that they are free from infection as the development of detectable antibodies following infection can take several months and also some infected animals fail to produce detectable antibodies (De Bocr *et al*, 1979).

In the past, the only control attempted for the eradication of the disease was the slaughter of all sheep in the area and a subsequent restocking policy. These approaches are extremely expensive and may not be cost effective as strict quarantine measures must be applied to avoid reintroduction of the disease. Lambing is thought

to contribute to spread of the disease and its discontinuance is recommended in infected flocks. In those countries where MVV is enzootic, severe restriction of inter-farm movement and outdoor housing are obvious aids for limiting the dissemination of the infection.

Today, control of MVV is based upon early identification of infected animals by periodic serological 'testing and culling' of these reactors and their offspring, even if the latter are negative at the time of the test (De Boer *et al*, 1979; Houwers *et al*, 1983). The recommended frequency of the serological tests is every 6 months, but may be reduced to once a year after two negative tests (Jensen and Swift, 1988). This method does not guarantee the complete elimination of all infected animals as a few may not seroconvert even if they harbour the virus, but at least it reduces the rate of infection to a minimum.

Prevalence of the disease may also be reduced by avoiding exposure of healthy animals to infected ones (Sharma, 1982). The progeny from infected dams should be weaned at birth and separately reared by artificial feeding. Either pasteurised milk or a milk substitute can be used for this purpose (Light *et al*, 1979; Houwers *et al*, 1983) and colostrum from infected dams is suitable after heat-treatment to destroy the virus infected cells (Lerondelle and Ouzrout, 1990).

Slaughtering of animals at an earlier age (Hugosson, 1978), in combination with the 'test and culling' policy could help to reduce losses in commercial breeding flocks, while mass hysterectomy of the flock with artificial rearing of the lambs or embryo-transfer technique needs to be evaluated more thoroughly before recommending their use.

Once a flock is declared free of MVV, care must be taken to avoid its reintroduction as this virus is a constant threat to the maintenance of free flocks.

1.1.10 DIAGNOSIS

Diagnosis of MVV infection may be complex due to the insidious nature of the disease, the likelihood of it being masked by other pulmonary diseases (such as pasteurellosis and SPA) or by other debilitating and wasting diseases, and finally because changes produced by the virus might be masked by invasion of lungs by multiple pathogens (Pritchard *et al*, 1984).

Pathological changes, although highly suggestive for MVV infection both macro- and microscopically, are not pathognomonic.

Cultivation of virus can be used for diagnostic purposes, but it takes a long time and cannot be used as a routine test. Viral isolation can be attempted from live animals by cocultivation of peripheral blood leukocytes usually on cell cultures of sheep choroid plexus, lung, testis or spleen (Dawson, 1980).

One of the characteristic features of this infection is the persistence of the virus in infected animals and, once developed, a permanent detectable humoral response. The persistence of the virus in the presence of circulating antibodies allows a diagnosis of MVV infection to be made by identifying either virus or specific antibodies in the blood (Cutlip *et al*, 1978, 1986). Examination of serum samples searching for specific antibodies can be used for diagnostic purposes both during the incubation period and in the clinical phase. Several serological methods can be used to identify virus carriers (Thormar *et al*, 1966; De Boer, 1970; Cutlip *et al*, 1977; Dawson *et al*, 1979; Houwers and Gielkens, 1979; Molitor *et al*, 1979; Winward *et al*, 1979; Biront *et al*, 1981; Cutlip and Lehmkuhl, 1986). Currently, the most frequently used techniques are the agar-gel-immunodiffusion test (AGIDT) and an enzyme linked immunosorbent assay (ELISA); both are equally specific for detecting antibodies to MVV in ovine sera after a comparative trial (Dawson *et al*, 1982). The simplicity of the AGIDT makes it the test of choice for most surveillance or control programs (Dawson *et al*, 1982), but the indirect ELISA developed by Houwers and Gielkens in 1979, seems to be slightly more sensitive than the AGIDT (Houwers *et al*,

1982) being able to detect infected sheep at an earlier stage post-infection. An indirect immunofluorescence test is also available (Dawson *et al*, 1982), but has not been widely used. Other serological tests that have been used are the complement fixation test (Larsen *et al*, 1982a), serum neutralisation test (Sigurdsson *et al*, 1960), microimmunodiffusion test (Cowan and Wagner, 1970), immunoelectro-osmophoresis (Pan *et al*, 1972) and a radial immunodiffusion test.

Serology, however, has some limitations. Passively acquired antibodies obtained through the colostrum are lost by six months of age making serological tests uninterpretable during the first six months of age. Due to this, the best application of serological testing will be on a herd basis.

Summarising, a definitive diagnosis depends upon finding characteristic lesions at necropsy in conjunction with clinical signs, information about the spread of the disease (Sharma, 1982) and demonstration of virus by either serology or isolation.

1.2. PULMONARY IMMUNE RESPONSE DURING LENTIVIRAL INFECTION: HIV AS A MODEL

1.2.1 HUMAN RETROVIRUSES AND LUNG DISEASES

In the last few years a wide spectrum of human pulmonary diseases has been associated with the local immunodeficiency induced by retroviruses (Rankin *et al*, 1988; Semenzato and Agostini, 1989).

At least three human retroviruses belonging to the *Oncovirinae* subfamily are currently known, namely human T-cell leukemia virus type I (HTLV-I) (Poiesz *et al*, 1980), HTLV-II (Rosenblatt *et al*, 1986) and HTLV-V (Manzari *et al*, 1987). HTLV-I infection accounts also for some neurodegenerative disorders; in the lung this virus shows a peculiar tropism for helper-related CD4⁺ T cells in which it produces partial activation of the interleukin-2 receptor (IL-2R) and interleukin-2 (IL-2) gene expression, this probably leading to a period of autocrine T-cell growth and finally to the ultimate leukemic transformation (Leung and Nabel, 1988).

Besides oncoviruses, two other human retroviruses evolutionarily related to the lentivirus family have recently spread across many parts of the world, becoming a dramatic threat to public health, i.e. HIV-1, the etiologic agent of acquired immune deficiency syndrome (AIDS) (Barre-Sinoussi *et al*, 1983) and the more recently isolated HIV-2 (Clavel *et al*, 1986).

HIV-1 shares morphology (Gonda *et al*, 1985), genomic sequence homologies (Gonda *et al*, 1985; Sonigo *et al*, 1985) and replication mechanisms (Hess *et al*, 1985; Davis *et al*, 1987) with the ungulate lentiviruses. Lung pathology is also similar in both viral infections, neonatal lambs inoculated with ovine lentivirus developing a lymphoid interstitial pneumonia which is morphologically and clinically similar to that of juvenile and adult AIDS (Lairmore *et al*, 1986). Furthermore, related pulmonary complications in both diseases are associated with similar abnormalities of



the BALF cellular constituents. This review will focus on what is currently known about the effects of HIV-1 infection on host lung defences, aiming to provide some insights into the mechanisms by which MVV operates in the ovine lung during MVV-infection.

1.2.2 PULMONARY IMMUNE RESPONSE AGAINST HIV-1

Studies performed on cells recovered by BAL, combined with the availability of monoclonal antibodies (MAbs), cell culture and recombinant cytokines and molecular probes, have allowed the investigation of those events leading to the progressive impairment of pulmonary defence mechanisms during HIV-1 infection.

In HIV-1 infection, pulmonary immunologic alterations are an early phenomenon. The main immunocompetent cell types usually found within alveolar spaces of patients with HIV are lymphocytes and alveolar macrophages (Venet *et al*, 1983; Agostini *et al*, 1988; Nowakowski *et al*, 1992). Polymorphonuclear leukocytes (PMNs), although present in normal numbers in asymptomatic patients, can be increased during concurrent opportunistic infection (Agostini *et al*, 1991a; Nowakowski *et al*, 1992).

1.2.3 ROLE OF PULMONARY LYMPHOCYTES

Lymphocytes participate in host defences by regulating other immune mechanisms (helper and suppressor T cells), by producing antibodies against pathogens (B cells) and by participating in killing of target cells (cytotoxic T lymphocytes (CTL) and natural killer (NK) cells).

1.2.3.1 T lymphocytes

In contrast to the lymphopenia usually observed in PB, which mainly reflects a depletion of circulating CD4⁺ cells, pulmonary lymphocytes are increased in patients with AIDS. The phenotypic evaluation of BALF T cell subpopulations shows an

increase in both the percentage and total number of lymphocytes, with alterations in lymphocyte subsets. The lymphocytic alveolitis in AIDS comprises mainly CD8⁺ cytotoxic T cells (Venet *et al*, 1983; Young *et al*, 1985; Agostini *et al*, 1988; Nowakowski *et al*, 1992). The pulmonary helper CD4⁺ lymphocytes are virtually absent, resulting in a marked decrease in the CD4⁺/CD8⁺ ratio.

Two different hypotheses have been proposed to account for the accumulation of lymphocytes within the lung during the course of HIV-1 infection, either a recruitment of these cells from the PB to the lung, or an IL-2 driven proliferation of preexisting T cells (Hunninghake *et al*, 1983; Pinkston *et al*, 1983; Semenzato, 1991). BALF T lymphocytes of HIV-1 seropositive patients, apart from expressing the IL-2R, (Zambello *et al*, 1992) bear some other activation markers on their surface such as MHC Class II antigens (HLA-DR) (Agostini *et al*, 1993b).

In addition to increased lymphocyte populations in the alveolar space, open lung and transbronchial biopsies from HIV-positive patients have shown that CD8⁺ T cells also infiltrate the pulmonary parenchyma (Beck and Shellito, 1989) and interstitium (Solal-Celigny *et al*, 1985). Peribronchiolar lymphocyte infiltration has also been observed (Ettensohn *et al*, 1988).

Lung CD8⁺ T cells of HIV-1-infected patients are represented by two different subpopulations of cytotoxic T lymphocytes: CD3⁺/CD8⁺/S6F1^{bright} CTL restricted by the MHC and CD3⁺/CD8⁺/S6F1^{bright} T-cells bearing NK markers (Agostini *et al*, 1990; Plata *et al*, 1990). While the former CD8⁺ T cell subpopulation is involved in the host defence mechanisms against HIV-1 (being able to recognise and attack HIV-1 antigens on the surface membrane of infected cells), the MHC-unrestricted subpopulation does not seem to take part in host defence mechanisms against HIV-1, such cells being likely to contribute to the resistance against the development of AIDS-related opportunistic infections (Rankin *et al*, 1988; Beck and Shellito, 1989; Semenzato, 1991).

Only in the more advanced phases of the syndrome is the absolute number of CD4⁺ cells severely reduced (Semenzato and Agostini, 1989), the number of CD4⁺ cells being higher in those patients with recent infection than in those with concurrent opportunistic infections.

Although the majority of cytotoxic cells in the HIV-1 infected lung coexpress the CD3 antigen and the $\alpha\beta$ TCR, some $\gamma\delta$ TCR cells are also found within the lung. These $\gamma\delta$ TCR lymphocytes differ phenotypically from those in PB, as a high proportion of them coexpress the CD8 antigen (Agostini *et al*, 1990, 1994). These $\gamma\delta$ TCR cells seem to play a cytolytic role in the lung (Spits, 1991), however, their exact functional role is still unknown. It is speculated that, by recognising different sets of HIV-1 antigens from those recognised by $\alpha\beta$ TCR cells, they could play a major role during the pulmonary immune response in AIDS (Agostini *et al*, 1993a, 1994).

Pulmonary NK cells from AIDS patients are quantitatively increased and maintain the capacity to bind sensitised targets (Agostini *et al*, 1988). However, they are defective, as they lose the ability to release the cytotoxic factor involved in the NK lytic machinery (Semenzato, 1991).

1.2.3.2 B lymphocytes

Humoral immunity may also be abnormal in pulmonary AIDS. BALF from AIDS patients shows increased total amounts of IgG, IgM and IgA (Young *et al*, 1985). The presence of increased quantities of albumin (a protein only synthesised in the liver) in BALF of patients with AIDS suggests that the integrity of the alveolar-capillary membrane is compromised and some serum proteins are passing into the pulmonary epithelial lining fluid (PELF) (Beck and Shellito, 1989). Although it is likely that some of the increase in BALF Ig is due to transudation from the intravascular space, local production by pulmonary plasma cells is also a probable

additional source (Young *et al*, 1985). This is supported by the finding of increased numbers of IgG, IgM and IgA secreting cells in AIDS patients (Rankin *et al*, 1983).

1.2.4 ROLE OF ALVEOLAR MACROPHAGES

Alveolar macrophages are important elements in host defence against pulmonary infection as they possess phagocytic and microbicidal properties. These cells can be infected *in vitro* by HIV-1 (Clarke *et al*, 1990; Plata *et al*, 1990; Rose *et al*, 1991). They also contain HIV-1 RNA (Plata *et al*, 1987), suggesting that they might serve as a reservoir for the virus and therefore, perpetuate the infection *in vivo*. Most of the alveolar macrophages within the lung from HIV-1 infected subjects derive from circulating monocytes (Holian and Scheule, 1990), although some of them can also replicate in the normal lung.

Apart from being a target for HIV-1, in the early stage of AIDS, alveolar macrophages could be involved in the host defence mechanisms against HIV-1 and against opportunistic infections (Reynolds, 1987; Agostini and Semenzato, 1990), and a macrophage alveolitis has been reported in some HIV-1 patients (Agostini *et al*, 1991a). Interesting phenotypic features are found in alveolar macrophages from HIV-1 infected patients. They appear to be in a permanent state of activation showing upregulated surface expression of CD25 antigen (p55 chain of the IL-2R) (Agostini *et al*, 1993b), MHC Class II (HLA-DR) antigens, adhesion molecules (CD44, CD54) and CD4 determinants. As CD4 is the receptor for HIV-1, its increased expression on alveolar macrophages could contribute to the HIV-1 infection of these cells. Reduced expression of some specific alveolar macrophages markers has also been reported (Bray *et al*, 1991).

Alveolar macrophages isolated from patients with AIDS release a series of cytokines. They constitutively release tumour necrosis factor α (TNF- α), a proinflammatory cytokine which might play a role in the pathogenesis of AIDS-

related pulmonary complications (Semenzato, 1990) and favour viral spread (Osborn *et al*, 1989; Krishnan *et al*, 1990; Agostini *et al*, 1991b; Israel-Biet *et al*, 1991; Millar *et al*, 1991a). However, the function of TNF as an inflammatory mediator can also be beneficial as it promotes endothelial adhesion by PMNs, induces the synthesis of IL-1 by macrophages and may have antiviral properties (Beutler and Cerami, 1987). Alveolar macrophages from HIV-1 patients also secrete increased amounts of IL-6 and IL-6 inhibitor (Millar *et al*, 1991b; Trentin *et al*, 1992) and of granulocyte macrophage-colony stimulating factor (GM-CSF) (Agostini *et al*, 1992). The regulatory network between HIV-1 and cytokines in the lung seems to be essential for the regulation of the HIV-1 production, latency and reactivation.

Reports about the effect of HIV-1 infection on the antimicrobial faculty of alveolar macrophages are contradictory. Hence, while some authors claim that HIV-1 does not affect the killing capacity of these cells (Murray *et al*, 1985), others have demonstrated some decrease in the microbicidal functions of alveolar macrophages from those AIDS patients who developed pneumonia (Musher *et al*, 1990); such an impairment might explain the spectrum of pulmonary pathogens encountered in AIDS patients (Beck and Shellito, 1989).

1.2.5 ROLE OF POLYMORPHONUCLEAR LEUKOCYTES (PMNs)

Like alveolar macrophages, PMNs, contribute to host defence by intracellular killing of organisms through chemotaxis, phagocytosis and subsequent generation of toxic metabolites. Their number increases in the last phase of AIDS when opportunistic infection of the respiratory tract occurs. Neutrophils are the main cell type which accounts for this increase (Agostini *et al*, 1991a), while eosinophils are only occasionally increased (Fleury-Feith *et al*, 1989). The increased lung neutrophils seems to be directed to the clearance of pulmonary pathogens, although the mechanisms for the recruitment of these cells into the lung of HIV-1 infected patients

is not yet fully understood. The macrophage-derived IL-8 seems to initiate and direct the migration of PMNs into the lung (Basha *et al*, 1991), while some other cytokines such as IL-3, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF), by promoting bone marrow differentiation and proliferation of neutrophil precursors, would enhance neutrophil accumulation.

1.3. BRONCHOALVEOLAR LAVAGE (BAL)

1.3.1 INTRODUCTION

The importance of the lung as a target organ in several infections and allergic diseases has focussed attention on the immune responses of the lower respiratory tract. The inflammatory states that can be found in the lungs are the result of the humoral and cellular interactions during the immune response. A much better understanding of the inflammatory processes occurring in human chronic lung diseases has been achieved by the use of the bronchoalveolar lavage (BAL) (Crystal *et al*, 1984).

BAL performed using a fiberoptic-endoscope has enabled sampling of the airway lumen cell population which consists mainly of macrophages and lymphocytes. The BAL sample represents principally an alveolar lining sample as the alveolar surface area is approximately 100 times greater than that of peripheral airways. The lymphocyte cell population harvested by this procedure correlates fairly well with cell populations obtained by lung biopsy and necropsy samples, and it has even been reported that the effector cells recovered by BAL are a more accurate reflection of the cell population in the lower respiratory tract than that obtained from biopsy tissue (Haslam *et al*, 1980).

1.3.2 BAL APPLICATIONS IN MAN: ROLE AS A CLINICAL, DIAGNOSTIC AND RESEARCH TOOL

BAL has been commonly used for the study of interstitial lung diseases, which are characterised by diffuse inflammation of the pulmonary parenchyma and interstitium. Since its introduction there has been debate regarding the application of BAL, as the boundary between its clinical and research function is not yet clear. Daniele and others (1985) examined this technique, concluding that the BAL is primarily a research procedure, having several limitations in the clinical site.

There are few complications associated with fiberoptic bronchoscopy (its ease and safety and the benign nature of lavaging different subsegments of pulmonary lobes) hence, BAL has become a widely acceptable means for recovering cells and proteins from the lower respiratory tract of subjects with many different respiratory diseases.

1.3.2.1 BAL AS A CLINICAL TOOL

The cell population recovered by BAL resembles in proportion, function and type that found in the alveoli regardless of the lobe lavaged. Because of the possibility of estimating the number of free cells in the lungs, BAL constitutes a very sensitive method for the detection of early pulmonary cellular responses, and for helping to stage and monitor the progress of the pulmonary disease. Other respiratory tract sampling procedures have more disadvantages than BAL and therefore, *repeated open lung biopsy* is unsuitable, associated with some morbidity, limited to the sampling of few alveoli, and presenting some difficulties in the specific identification of infiltrating cells; *transbronchial biopsy*, apart from its significant sampling error is contraindicated in some patients, because of the risk of causing pneumothorax, haemorrhage and even death; *chest radiographs and standard pulmonary function tests* are not completely satisfactory for monitoring disease evolution.

1.3.2.2 BAL AS A DIAGNOSTIC TOOL

By permitting the exploration of previously inaccessible distal airways BAL has made possible the diagnosis of respiratory processes by identification of cellular constituents, recognition of inorganic molecules inside alveolar macrophages and detection of abnormalities in local cellular immune responses. It has also proved to be particularly useful for the diagnosis of opportunistic infections (for instance the diagnosis of pneumonia due to opportunistic infections such as *Pneumocystis carinii*, especially in patients with AIDS).

However, it does not constitute a very valuable aid for the diagnosis of bacterial respiratory infection as it is technically difficult to collect BALF without oropharyngeal bacterial contamination.

1.3.2.3 BAL AS A TOOL FOR PULMONARY RESEARCH

Knowledge about the role of airway cells and secretions in the immunopathogenesis of lung diseases has improved and, hence, BAL has recently acquired a crucial importance in pulmonary research.

It enables study of the immune properties and functions of the harvested cells; MAbs have been used to characterise antigens on the surface of these cells, permitting flow cytometric analysis for assessing the subpopulations of the recovered cells. Furthermore, it also allows a qualitative and quantitative study of the lavage supernatant components, as a single analysis of lavage is quite representative of all the cells and acellular constituents throughout the lung (Crystal *et al*, 1986).

1.3.3 STANDARD METHOD

Optimally, sampling procedures should meet the following criteria (Saltini *et al*, 1984): they should be easy to perform, produce a reliable recovery and precise quantification of all the cell types present, allow routine histological and histochemical staining procedures, yield specimens that can be stored indefinitely and provide cells in good morphological condition.

BAL, usually performed by fiberoptic endoscopy, meets all these requirements. To avoid blood contamination, which could modify cell and protein values, the lavage must be done prior to any bronchial brushing or transbronchial biopsy.

It is desirable to lavage multiple lung areas as there may be slight lobar differences in cell counts. In human patients one wash from each lung is usually

performed and the respective specimens are assayed independently. Although usually both samples are quite similar in normal patients, in the diseased lungs some variations may be appreciated (Nugent *et al*, 1984).

Physiological sterile saline solution is infused through the channel of a fibreoptic endoscope (previously wedged into a midsized bronchus). A medium without Ca^{++} and Mg^{++} ions is preferred for the washing as it decreases cell clumping and provides a better yield of cells and proteins.

Warming the saline to 37°C will slightly increase the harvest of respiratory cells (Pingleton *et al*, 1983) and may reduce coughing.

The saline solution 'washes out' the material lining the epithelial surface of the bronchial tree and alveoli distal to the bronchoscope, termed the PELF, which is then recovered by gentle suction. Excessive suction will reduce cell viability (by damaging the recovered cells) and could also cause a collapse in the airways distal to the tip of the bronchoscope, decreasing the effective return of the fluid.

The time the lavage fluid must remain in the lung before aspiration (termed the 'dwell time') has not been well established, but it seems that a better sample is obtained when the patients breathe once or twice before the extraction of the fluid. Rennard and others (1986), however, have shown that as the 'dwell time' increases the diffusion of molecules, i.e., histamine, urea, albumen from the blood and lung into the BAL fluid also increases and may lead to incorrect measurement of these molecules.

The frequent presence of foam in the later samples denotes that surfactant is present, indicating therefore that the site where the recovery is taking place includes the alveoli.

It is advisable to discard the first wash as it differs from all the other subsequent washes, not only because of its smaller volume but also because of the fact that it represents more a bronchial than an alveolar sample. Besides, the content of epithelial cells in this first fraction is usually relatively high.

Any mucus or debris recovered with the fluid can be easily eliminated along with some of the surfactant froth by filtering the specimen through a single layer of loose cotton gauze.

1.3.4 ANALYSIS OF RECOVERED CELLS

Firstly, the number and type of the recovered cells are determined. For this, cells in the neat lavage fluid (without any previous dilution or concentration) are counted in either an haemocytometer chamber or in a Coulter counter and expressed as cells per millilitre or μl of solution. Differential cell counts are performed on cytocentrifuged preparations stained with Leishman's stain or any other analogous stain (i.e. Wright Giemsa). Occasionally special staining techniques may be required to assess the identity of certain cell types.

1.3.5 PROBLEMS ASSOCIATED WITH BALF PROCESSING

Different methods for processing BALF samples can induce variations in the results of lavage analysis (Mordelet-Dambrine *et al*, 1984; Daniele *et al*, 1985) and therefore, these technical problems must be considered when comparing BAL cell counts published by different groups.

By analysing several criteria, Mordelet-Dambrine and others (1984), concluded that the *centrifugation speed* and the *number of cell washes* were the main parameters influencing differences in cell count. The reading of the preparations by different investigators along with the utilisation of different haemocytometers did not cause a significant variation in the cell counts. On the other hand, the absolute cell count was higher before washing and higher centrifugation speeds led to a loss of macrophages.

Due to the inevitable loss of cells during centrifugation (affecting all the cell types) the quantification should be performed on the neat lung fluid prior to centrifugation. Discrepancies in the number of cells recovered will also depend on the

volume of fluid instilled and recovered (Daniele *et al*, 1985). Another feature to be considered is that different fractions of lung fluid always differ in their predominant cell types (Davis *et al*, 1982; Martin *et al*, 1987); the first fraction containing more PMNs and fewer macrophages, while the later fractions will be richer in lymphocytes.

Saltini and others (1984) and Walters and Gardiner (1991) have reported that the cytocentrifuge technique underestimates the percentage of recovered lymphocytes. In order to prevent this lymphocyte loss and whenever a very accurate quantification of cells is required, the same authors have described a filter preparation technique which also provides a good morphologic detail of cells; this procedure tends to collect all the cell populations prior to staining. This technique however is much more time-consuming.

Additionally, coughing or expectoration during the performance of the BAL could affect the results.

1.3.6 BAL IN SHEEP

The sheep lung, due to its anatomical features, has been proposed as an excellent model for the study of pulmonary immune responses (Joel and Chanana, 1985). Most studies on immunity of the respiratory tract of sheep had been limited to the upper respiratory tract. The use of the fibroptic endoscope has allowed the study of the immunity of the lower respiratory tract in normal sheep (Gorin and Steward, 1979; Rola Pleszczynski *et al*, 1981). Some work has also been done in diseased sheep; Burrells (1985a) reported quantitative, qualitative and functional differences in BALF from sheep inoculated with *Mycoplasma ovipneumoniae*, *Pasteurella haemolytica* and parainfluenza-3 virus, when compared with control animals. Cordier and others (1990) described increased BALF cellularity, increased percentage of neutrophils and activation of alveolar macrophages in BALF from MVV-infected sheep.

Although BAL has been used in unsedated cattle (Fogarty *et al*, 1983) and sheep (Begin *et al*, 1981), the introduction of catheters or of the fiberoptic bronchoscope into the airways of conscious sheep is not well tolerated (Rola Pleszcynski *et al*, 1981; Williams and Burrells, unpublished observations), since it is invariably associated with marked spontaneous coughing. This cough could lead to small capillary blood vessel rupture and hence to blood contamination of the lung fluid recovered making it inappropriate for humoral or cellular studies. Due to the disadvantages of performing the lung lavage in conscious animals, two alternative methods for the recovery of respiratory fluid in sheep have been described (Burrells and Williams, 1986), namely gross lavage of excised lungs and segmental lavage on anaesthetised sheep (either by endoscopic catheterisation or by fiberoptic bronchoscopy).

1.4. FLOW CYTOMETRY

1.4.1 INTRODUCTION

The flow cytometer is a sophisticated apparatus for cell analysis. It uses laser light sources and sensitive optics that can analyse cells on the basis of light scattering and fluorescent properties and thus, improve the degree of accuracy with which cells from lavage fluid are identified. A suspension of cells appropriately labelled with fluorochrome conjugated antibodies to cell surface antigens is projected through the nozzle of the machine under pressure. Each cell, on passing the light beam, generates optical signals by scattering the light or emitting fluorescence and these optical signals are detected by photodetectors (lenses and optical filters) which convert them into electrical signs relating cell volume to its electronic impedance. These electronic signals are then stored and processed by a computer.

Three different parameters are usually recorded for each cell: forward angle light scattering or forward scatter (FSC), which represents the light that is deflected out of the laser beam through a small angle ($0-10^\circ$) and is related to cell diameter; right angle light scattering or side scatter (SSC), which is the light that is deflected by the cells through a wide angle ($70-110^\circ$) and gives information about the internal structural properties of the cells (i.e. those cells with large number of cytoplasmic granules or other organelles scatter more light than erythrocytes or lymphocytes); and fluorescence, related to binding of fluorochrome.

The simultaneous analysis of two of the variables, FSC and SSC, permits the rapid identification and selective analysis of the particular population of cells one wishes to analyse. Furthermore, the use of the FSC allows discrimination between viable and non-viable cells since dead cells give rise to a smaller forward scatter signal than live cells (Loken and Stall, 1982). By using a third parameter, the binding of fluorochromes, the phenotypic expression of the cells can be identified.

1.4.2 ANALYSIS AND DISPLAYS

The most common and useful forms of display are the 'frequency histogram' and the 'dot plot' or dual parameter correlated dot plot. Data can also be presented in other forms such as the 'three-dimensional histograms' (contour maps or contour plots).

The frequency histogram or single parameter histogram is the simplest form of data display available. In this histogram either units of fluorescence intensity or light scattering per cell are plotted on the horizontal axis which usually consists of 256 channels, and frequency or number of cells on the vertical axis. The conventional manner for analysis of an immunofluorescence histogram is to separate positive and negative populations and to set a threshold or gate, those cells with fluorescence intensity above the threshold being considered to show positive labelling. To set this 'fluorescence threshold' an appropriate control is used as reference; this gate is usually selected such that only a small percentage of control cells are scored as positive.

In the dot-plot each cell appears on the screen as a dot whose X and Y axis coordinates represent the light scatter and fluorescence signal for the cell. By inspection of such a plot it is easy to gain an impression of which size of cells bears the fluorescent label. However, by using the dot-plots is not possible to assess visually the quantitative distribution of the various cell populations as all cells that fall in a particular location will be represented only by a single dot. Three dimensional histograms show frequencies of cells for all pairs of red and green intensities (Herzenberg, 1978).

1.4.3 POTENTIAL ADVANTAGES OVER METHODS EMPLOYING FLUORESCENCE MICROSCOPIC ANALYSIS

Flow cytometry enables rapid, multiparameter analysis of much larger numbers of cells than fluorescence microscopy, avoiding tedious visual counting. It provides quantitative data, allowing an accurate measure of the relative amount of

antibody bound to each cell (this is of great relevance as a cell population is often identified on the basis of the amount of a particular marker rather than simply on its presence or absence). It is also much more sensitive, being able to detect very low levels of fluorescence. Finally, it offers the possibility of characterising cell populations and subpopulations providing a less subjective interpretation of the results than fluorescence microscopy.

1.5. SHEEP LEUKOCYTE MOLECULES IDENTIFIED BY SPECIFIC MONOCLONAL ANTIBODIES

1.5.1 INTRODUCTION

Most of the molecules on the surface of ovine leukocytes and defined by MAbs, are homologous to human or rodent molecules; many mammalian leukocyte molecules have conserved their structure, function and tissue distribution during evolution (Mackay *et al*, 1988b). The review below is a summary of those sheep leukocyte molecules most relevant to this study.

1.5.2 CD45 (LCA)

The CD45 molecule of mammals (formerly LCA or T-200) is a panleukocyte marker. It consists of a group of integral membrane glycoproteins, ranging in molecular weight from 180 to 220 kDa, which are expressed only on cells of the haematopoietic lineage (either immature or mature), including T and B cells, thymocytes, mononuclear phagocytes and PMNs. Isoforms of CD45 proteins that are expressed on a restricted group of cell types are designated CD45R. CD45R expression on T cells is of interest because it is developmentally regulated during the maturation process of T cells and hence, naive T cells express a form of CD45R called CD45RA, while memory T cells express a different isoform called CD45RO.

In sheep, CD45 is expressed on the surface of all lymphocytes of the thymus, spleen, lymph nodes, ileal Peyer's Patches and PB, as well as being found on macrophages and PMNs (Maddox *et al*, 1985a). Three different epitopes of the LCA of sheep have been characterised, one of which is expressed only by B cells and a unique lymphocyte subset (Mackay *et al*, 1987). The MAb 1-11-32 identifies a determinant present on all sheep leukocytes which had the molecular characteristics expected of the sheep analogue of rat, human and mouse LCA. Another MAb 20-96,

produced against the p220 component of sheep CD45, revealed a restricted tissue distribution, reacting with all B cells and a subpopulation of CD4⁺, CD8⁺, T19⁺ and sIg⁺ lymphocytes (Mackay *et al*, 1987). Different cell lineages or cells at various differentiation stages express different molecular forms of LCA, which result from differential processing of a primary mRNA transcript from one LCA gene (Saga *et al*, 1986). The sheep CD45 presents a 190 kDa molecule on B cells from ileal Peyer's Patches in contrast with peripheral B cell CD45 which is 220 kDa (Mackay *et al*, 1987).

The precise function of this molecule is still unknown, although MAbs reactive with LCA inhibit B cell differentiation (Yakura *et al*, 1983), accessory cell function (Mairo *et al*, 1981), NK cell lysis (Newman *et al*, 1983) and lysis by cytotoxic T lymphocytes (Harp *et al*, 1984).

1.5.3 CD2 AND LFA-3

CD2 (also referred to as T11, Leu-5 or Tp50) is the sheep erythrocyte receptor (Hunig *et al*, 1986). It is a 40 kDa surface glycoprotein that is present on approximately 90% of mature T cells, 50-70% of thymocytes (appearing early on stage I thymocytes) and on NK cells

CD2 functions as an intercellular adhesion molecule, and an identified ligand for CD2 is the structurally similar molecule called leukocyte function associated antigen-3 (LFA-3) or CD58 (Denning *et al*, 1988). LFA-3 is a 55 to 70 kDa surface glycoprotein expressed on a wide variety of haematopoietic and nonhaematopoietic cells. CD2 binding to LFA-3 promotes cell-cell adhesion. In addition to its adhesive function, CD2 is also a signal-transducing molecule, being able to facilitate T-lymphocyte target cell interactions and to activate resting T-lymphocytes (Hunig *et al*, 1987). CD2 may activate T cells without MHC and antigen interaction, which might potentiate immune responses in the absence of additional antigen stimulation (Meuer *et al*, 1984).

Only one MAb, namely 36F, is specific for ovine CD2 (Mackay *et al*, 1988a; Hein *et al*, 1991).

1.5.4 LFA-1 (CD11a/CD18)

Leukocyte function associated antigen-1 (LFA-1) is a member of the integrin family of heterodimeric leukocyte surface proteins (Arnaout, 1990), which function primarily as adhesion molecules. LFA-1 is expressed on virtually all bone marrow-derived cells, including more than 90% of thymocytes and mature T cells, B cells, PMNs and monocytes (Springer *et al*, 1987; Kishimoto *et al*, 1989).

Anti-LFA-1 antibodies inhibit a wide variety of adhesion-dependent lymphocyte functions (Arnaout, 1990), including antigen and antigen presenting cell-inducer helper T cell stimulation (Hildreth and August, 1985), CTL-mediated killing of target cells (Arnaout *et al*, 1984), and lymphocyte adhesion to high endothelial venules (Haskard *et al*, 1986). Intercellular adhesion molecule-1 (ICAM-1), an 80-114 kDa integral membrane glycoprotein is the specific ligand for LFA-1 (Springer *et al*, 1987).

Six MAbs have been described as CD11a specific. All these MAbs react with a 180,000 MW α chain associated with the CD18 β chain (β_2 -integrin) (Hopkins *et al*, 1993a).

1.5.5 CD25 OR IL-2R

This single chain glycoprotein, often referred to as the α -chain of the interleukin 2-receptor (IL-2R) or the Tac antigen, has a MW of 55 kDa (Robb *et al*, 1984), and is present on activated T (Yagita *et al*, 1989) and B (Waldmann *et al*, 1984) cells, activated macrophages (Hancock *et al*, 1986) and stimulated monocytes (Holter *et al*, 1987; Allen *et al*, 1990), playing a key role in antigen-induced proliferation. Together with the β -chain of the IL-2R (p75, MW 75 kDa), the CD25

antigen forms a high affinity receptor complex for IL-2 (Robb *et al*, 1984; Teshigawara *et al*, 1987).

1.5.6 CD3 AND TCR

The CD3 molecule in man and mice is a pan T-cell antigen closely linked with the T cell antigen receptor (TCR). It appears early and is retained throughout T-cell maturation. The CD3 molecule seems to act as a signal transducer for the TCR, leading the CD3/TCR interaction to a cascade of intracellular reactions that are fundamental for cellular activation (Oettgen and Terhorst, 1987). Sheep CD3 has been characterised using a rabbit anti-human CD3 antiserum which crossreacts with the sheep molecule; however, no MAbs against this molecule have been raised so far.

1.5.7 CD5

CD5 (also referred to as T1, Tp67 or Leu-1 in humans and Ly-1 in mice) is a pan-T cell antigen which appears early on thymocytes and is retained throughout cell maturation. In man, rat, mouse and sheep this antigen consists of a single glycopolypeptide chain with a MW of 67 kDa (Mackay *et al*, 1985; Beya and Miyasaka, 1986). The tissue distribution of ovine CD5 is analogous to that reported for CD5 in other species (Mackay, 1988). All T cells, thymocytes and some B cells express this molecule, medullary thymocytes expressing higher levels of CD5 than most cortical thymocytes (Mackay *et al*, 1985). In sheep, CD5⁺ B cells are abundant in adult spleen (representing up to 20% of B cells) but are not detected within lymph nodes or efferent lymph, suggesting that these cells might not be recirculating (Mackay, 1988).

The functional role of the CD5 molecule is still unknown, although MAbs directed against the CD5 antigen potentiate T cell proliferation in response to antigens and mitogens (Dallman *et al*, 1984) and cause an increase in the production of IL-2 (Ledbetter *et al*, 1985). CD5 has been associated with the execution of T helper

functions of CD4⁺ cells and with positive regulatory mechanisms involving T cell proliferation (Srouf *et al*, 1980). Although the ligand for CD5 has not yet been determined, similar structural features to other human receptor molecules, such as the insulin-receptor, IL-2R and transferrin receptor have been attributed to this ligand, due to the high conservation of mammalian leukocyte molecules through evolution (Mackay and Mackay, 1989). CD5, identified in sheep by the 25-91 MAb (Mackay *et al*, 1985) is, so far, the best pan T cell marker for sheep (Mackay *et al*, 1987).

1.5.8 CD4 AND CD8

The primitive thymocyte acquires both CD4 and CD8 antigen. As the thymocyte matures two separate T subpopulations emerge: one expressing CD4 but not CD8 (T-helper cell) and the other expressing only CD8 (T-suppressor cell). Double labelling of ovine T lymphocytes with SBU-T4 and SBU-T8 have shown that the populations recognised are mutually exclusive (Bujdoso *et al*, 1989).

CD4: CD4 antigen is a 55 to 62 kDa single chain cell membrane glycoprotein expressed on most thymocytes and on T cells in all mammalian species so far examined (Crocker *et al*, 1987). In sheep and cattle, CD4⁺ cells are functionally and structurally similar to those described in humans, rats and mice (Maddox *et al*, 1985b; Baldwin *et al*, 1986; Lalonde *et al*, 1986). CD4 is a non-lineage restricted molecule (Crocker *et al*, 1987), having been demonstrated on CD8⁺ T cell, cortical thymocytes, most medullary thymocytes, some B cell lines (transformed B cells), glial cells, dendritic cells and even on rat macrophages and human monocytes.

CD4 acts as a receptor for the invariable part of the HLA-class II antigens (Maddox *et al*, 1985b) and is involved in the induction of T-lymphocyte helper functions and T-proliferative responses to antigen stimulation. It also acts as a coreceptor for TCR2 ($\alpha\beta$ T cell receptor) in T cell activation (Hopkins *et al*, 1993a).

Additionally, the CD4 antigen is an important part of the receptor for HIV (Maddon *et al*, 1986).

MAbs produced to the sheep CD4 molecule, namely 44.38 and 44.97, do not crossreact with CD4 molecules in other species (Mackay *et al*, 1986).

In man, T cells expressing CD4 show restricted recognition of antigen in association with MHC class II (Marrack *et al*, 1983) and have mainly helper/inducer functions (Swain, 1983). Recent studies have discovered the functional heterogeneity of the CD4⁺ T cell subset as it includes helper and inducer lymphocytes as well as suppressor and cytotoxic cells (Thomas *et al*, 1983). On the basis of lymphokine secretion patterns the CD4⁺ T cells subset has been classified into another two categories, T_{H1} and T_{H2} (Janeway, 1975; Marrack and Kappler, 1975; Kim *et al*, 1985; Francien *et al*, 1988). The T_{H1} subset produces IL-2, γ -IFN and lymphotoxin, has cytotoxic capacity, suppresses some antibody responses, mediates macrophage activation and may also present helper activity for polyclonal B cell differentiation. The T_{H2} subset is mainly characterised by the production of IL-4, IL-5, IL-6 and IL-10, is a potent B cell activator and provides antigen-specific help (Kim *et al*, 1985; Tite *et al*, 1985; Mossman *et al*, 1986; Coffman *et al*, 1988).

CD8: CD8 in sheep is a glycoprotein which is a homodimer of 36 kDa on peripheral lymphocytes, existing in two forms, 33 and 36 kDa, when isolated from thymocytes (Maddox *et al*, 1985b). This molecule has a similar nature and tissue distribution to that of other species (Maddox *et al*, 1985b; Ezaki *et al*, 1987). This antigen is expressed on 10-25% of peripheral blood lymphocytes, 12-23% of efferent lymphocytes, on most cortical thymocytes and on CD4⁺ medullary thymocytes. Although also expressed by some NK cells in humans and rodents, this has not yet been investigated in sheep.

T cells expressing CD8 show restricted recognition of antigen in association with MHC Class I (De Vries *et al*, 1989), and have mainly cytotoxic functions

although a CD8⁺ CD5⁻ population clearly different from the classical suppressor/cytotoxic T lymphocytes has been observed in sheep (Meeusen *et al*, 1988) and also a subset of CD8⁺ T cells with an auxiliary role in adhesion has been described (Manakata *et al*, 1988). The function of sheep CD8⁺ T cells has also been studied *in vitro*, resembling that of other species in that anti-CD8 MAbs are able to block cytotoxic T lymphocyte function (Ezaki *et al*, 1987). Ovine CD8 also acts as a coreceptor molecule in T cell activation (Hopkins *et al*, 1993a). Several MAbs in sheep recognise CD8, namely 24.9 and 38.65 (Maddox *et al*, 1985b; Mackay *et al*, 1986), S-T8 (Ezaki *et al*, 1985; 1987), CC58 and IL-A51 (Keech and Brandon, 1991) and CC63 and BAT82A (Hopkins *et al*, 1993a).

1.5.9 $\gamma\delta$ T CELLS

$\gamma\delta$ T cells neither express B cell markers, nor are associated with B cell lymphoid tissue. They show a marked variance in tissue distribution between different species. In sheep, 30-60% of T cells express $\gamma\delta$ TCR, the number and localisation of these cells varying with age and appearing in the thymus during early foetal ontogeny (Maddox *et al*, 1987). Some $\gamma\delta$ T cells express a unique surface molecule termed T₁₉ (Brenner *et al*, 1986; Weiis and Imboden, 1987; Janeway *et al*, 1988; Mackay *et al*, 1989) which is antigenically unrelated to either CD45 or the TCR and without a known human or murine counterpart. The ovine T₁₉ molecule is a 215 kDa single chain polypeptide defining a lymphocyte subset which is CD2⁻, CD3⁺, CD4⁻, CD8⁻, sIg⁻, LFA-1^{low}, MHC Class I⁺ and CD5^{low}. T₁₉ is a maturation marker for $\gamma\delta$ T cells, appearing on nearly mature or mature $\gamma\delta$ thymocytes and playing a major role in their interaction and function (Mackay *et al*, 1989). T₁₉ could also be responsible for the homing pattern of $\gamma\delta$ T cells. *In vitro* activated $\gamma\delta$ T cells, such as those from the gut epithelium, express less T₁₉ than resting $\gamma\delta$ T cells (Mackay *et al*, 1991). The distribution of T₁₉ cells in peripheral lymphoid organs is exclusively restricted to the small lymphocyte population, exhibiting the characteristics of normal recirculating

lymphocytes and being responsive to T cell mitogens but not to B cell mitogens (Mackay *et al*, 1986).

$\gamma\delta$ T cells do not recognise antigens presented by MHC Class I or Class II encoded proteins, and the ligand for $\gamma\delta$ TCR has not yet been identified (Haas *et al*, 1990), although it is known that they use an adhesion molecule distinct from CD2.

The functional properties of $\gamma\delta$ T cells are not yet clear, but their location predominantly in epithelial surfaces suggest they could be a first line defence for the elimination of infected or transformed epithelial cells, and are apparently recruited to sites of active inflammation. *In vitro* experiments have also suggested some cytotoxic activity. (Hein and Mackay, 1991). Other possible roles that have been suggested for $\gamma\delta$ T cells are involvement in normal pregnancy and thymocyte development (Janeway *et al*, 1988).

Several MAbs specific for the T₁₉ molecule on sheep and cattle have been produced, namely 86D (Mackay *et al*, 1989), SBU-T19, 197, IL-A29, CC-15 and CC-39 (Hein *et al*, 1991).

The reactivity of MAb 86D, one of the markers used in our studies, is very similar or identical to that of anti- $\gamma\delta$ MAbs described in other species (Mackay *et al*, 1989). However, whether or not MAb 86D recognises all $\gamma\delta$ T cells is questionable. A $\gamma\delta$ lymphocyte subset in bovine PB has been shown to be T₁₉⁺ 86D⁻ (Mackay and Hein, 1989).

Results obtained from the first workshop on ruminant leukocyte differentiation molecules placed all the anti-T₁₉ MAbs into workshop cluster WC1, and MAbs to the $\gamma\delta$ TCR into workshop cluster WC2 (Hein *et al*, 1991; Howard *et al*, 1991). MAb CC15 recognises all WC1 in the sheep (Davis *et al*, 1990), while 86D is the MAb that characterises the WC2 (Mackay *et al*, 1989).

1.5.10 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) ANTIGENS (OVINE LEUCOCYTE ANTIGENS, OLA)

The MHC is a region of highly polymorphic genes whose products are expressed on the surface of a variety of cells (Hammerling *et al*, 1975; David *et al*, 1976). This locus was discovered in the 1940's during the transplantation of tissue from one individual to another. The MHC-encoded proteins are commonly referred to as 'MHC molecules' or 'MHC antigens'. They are involved in the regulation of T and B lymphocyte differentiation, in the ability of the host to mount humoral and cell mediated immune responses and in the basic cell-cell interactions that maintain the integrity of the immune system (Puri *et al*, 1987). There are two types of MHC gene products namely Class I and Class II. MHC antigens in the sheep are designated ovine leukocyte antigens (OLA) (Puri *et al*, 1985).

MHC Class I antigens in man and many other species are a series of membrane glycoproteins described as the classical 'transplantation antigens' (Gogolin-Ewens *et al*, 1985). They are found on the surface of peripheral blood leukocytes (Brown *et al*, 1979) and of many other cells (Fleming *et al*, 1981; Muller *et al*, 1983; Daar *et al*, 1984). Their main function is the recognition of foreign antigens by cytotoxic T-lymphocytes and the direction of cytotoxic effector cells against virus-infected targets (Zinkernagel and Doherty, 1974).

MHC Class II antigens are heterodimeric cell surface glycoproteins consisting of an α -chain and a β -chain (Schwartz and Cullen, 1978). These antigens are constitutively expressed by B lymphocytes (Sachs and Cone, 1973), monocytes (Winchester *et al*, 1976), certain haematopoietic precursor cells (Winchester *et al*, 1977), and Langerhans and dendritic cells (Rowden *et al*, 1977). Expression of MHC Class II antigens can also be induced on a variety of cell populations including T lymphocytes (Frelinger *et al*, 1974), macrophages (Steinman *et al*, 1980; Steeg *et al*, 1982), endothelial cells (Pober *et al*, 1983) and epithelia (Barclay and Mason, 1982).

Sheep Class II molecules have a tissue distribution very similar to that of man (Daar *et al*, 1984) and mice (Natali *et al*, 1981). Class II molecules are involved in humoral immune responses to soluble and cellular antigens (Kappes and Strominger, 1988), and have a major role presenting processed antigen to T cells.

Sheep Class II molecules were first described by Millot (1978), and later by Puri and others (1985) and Hopkins and others (1986), using a panel of MAbs. In man they are encoded by three distinct loci DP, DQ and DR and molecular analysis and sequencing data have identified a further two subregions, DN (Trowsdale and Kelly, 1985; Bodmer *et al*, 1988) and DO (Tonnelles *et al*, 1985). In the sheep they appear to be controlled only by two linked polymorphic loci, DR and DQ (Scott *et al*, 1987; Puri and Brandon, 1987; Dutia *et al*, 1990a; 1990b).

1.6 PLAN OF THESIS

This work has combined the use of sheep leukocyte MAbs with flow cytometry and BAL for the study of MVV-induced lymphoid interstitial pneumonia. The primary pulmonary pathology induced by MVV is characterised by a mononuclear cell infiltration of interalveolar septae (Watt *et al*, 1992). As the types and functions of cells in BALF reflect their counterparts in the pulmonary interstitium (Haslam *et al*, 1980), the technique of BAL can be used to monitor *in vivo* lung pathology during MVV-infection and to provide insight into cellular immune events in the normal and diseased ovine lung. The use of MAbs to sheep leukocytes and flow cytometry will facilitate characterisation of the phenotypes of both BALF and PB leukocytes. Furthermore, it will allow qualitative and quantitative comparisons of leukocyte subsets in BALF with those in PB, both in health and during the course of MVV-infection.

All the MAbs used for this work had been previously characterised. When this work started, no MAbs specific for macrophages had been characterised and hence, most of the MAbs employed were lymphocyte markers. Only markers of activation were employed on BALF macrophages.

MAb VPM18 (Hopkins and Dutia, 1990), the ovine homologue of the human CD45 molecule was used in this study as a positive control for leukocyte staining, as it is absent from non-leukocytic cells.

Staining with VPM8, the anti-ovine light chain specific MAb (Jones, 1988), was used to characterise B cells, as B cells are classically defined by the expression of surface immunoglobulin.

17D.13 (Mackay *et al*, 1988b) and SBU-T8 (Maddox *et al*, 1985b) were MAbs against the two main T lymphocyte subsets, CD4 and CD8 respectively. They were included to characterise, respectively, T helper and T cytotoxic/suppressor cells, and later to calculate the CD4⁺/CD8⁺ ratio. The CD4⁺/CD8⁺ ratio can be considered as an important measure of the immunoregulatory status (Hoffman *et al*, 1980).

Two MAbs which characterise $\gamma\delta$ T cells, namely 86D (Mackay *et al*, 1989) and CC-15 (Mackay *et al*, 1991) were included in this work because previous studies in man had suggested a role of these cells in the pulmonary immune response (Agostini and Semenzato, 1990).

To elucidate the possible role of immune cells in the pathogenesis of maedi, assessment of the activation status of those cells was essential. Therefore, several markers of activation were employed. F10/91/43, an anti-CD5 MAb, was used as a parameter of lymphocyte activation, as loss of CD5⁺ has been reported to occur during lymphocyte activation in the sheep (Hopkins and Dutia, 1990). MAbs against MHC Class II antigens with -DR and -DQ specificity were used in single colour flow cytometric analyses as markers of activation both on BALF and PB lymphocytes and on BALF macrophages. They were also employed in double colour flow cytometric analyses, combined with MAbs for the different T cell subsets. This allowed the characterisation of the differential pulmonary expression of DQ and DR by the different T cell subsets. Two pan MHC Class II MAbs, namely SW73.2 (Hopkins *et al*, 1986) and VPM36+VPM38, a combination of a - α DR (VPM36) and a - α DQ (VPM38) anti-MHC Class II MAbs were employed. VPM37 β and VPM54 α were the anti-MHC Class II MAbs with DR specificity used, and VPM41 β and VPM36 α those with DQ specificity (Dutia *et al*, 1990a; 1990b). Also as activation markers, two MAbs, F10/150/39 (Mackay *et al*, 1990) and L180/1 (Hunig *et al*, 1986), which characterise the adhesion molecules LFA-1 and LFA-3 respectively, were applied both

to BALF and PB lymphocytes and to BALF macrophages. Finally, IL-A111, an anti IL-2R α MAb (Naessens *et al*, 1992), was included in this study with a double purpose: firstly, to measure the activation state of BALF and PB lymphocytes and, secondly, to study the proliferative response of these lymphocytes to mitogen-stimulation.

CHAPTER 2: GENERAL MATERIAL AND METHODS

2.1. EXPERIMENTAL ANIMALS

The MVV-infected animals were Texel sheep, purchased from a Scottish commercial sheep flock as previously described (Watt *et al*, 1990; 1992a). The original flock had been established between 1972 and 1979 from 30 pedigree Texels imported from France. All animals purchased were seropositive to MVV, as determined by an agar gel immunodiffusion test (AGIDT) (Winward *et al*, 1979). Although the flock was maintained at grass, prior to the experimental period, animals were housed in individual pens for variable lengths of time.

Uninfected breed, age, and sex-matched control sheep were obtained from the Marshall Building, University of Edinburgh. They were housed in a single pen.

To eliminate gastrointestinal worms and lungworms, both groups of animals were treated every two months with oral ivermectin (Oramec Drench; Merck Sharp & Dohme, Hoddesdon, UK) at the recommended dose (2.5 ml/10 kg body weight). Housed animals were fed *ad libitum* on hay and water with a regulated supply of concentrates.

2.2. CLINICAL EXAMINATION OF THE ANIMALS

Animals were examined thoroughly using standard clinical techniques, faecal examination for lungworm larvae, routine haematology and clinical biochemistry procedures.

The respiratory system was studied with particular emphasis; sheep were examined for coughing, nasal discharge and dyspnoea and lung fields were auscultated. Thoracic radiography and pulmonary function studies were also performed.

2.3. ANTIBODY ASSAY

Serum from all sheep was examined for specific precipitating antibodies to MVV by the AGIDT (Winward *et al*, 1979). The viral antigen for the AGIDT was prepared from ovine lung cultures persistently infected with virus strain WLC-1, from the Central Veterinary Laboratory (Weybridge, UK). The immunodiffusions were examined at 24 and 48 h. The test was repeated in inconclusive cases.

2.4. COLLECTION OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

Transendoscopic bronchoalveolar lavage (BAL) was performed under general anaesthesia using an Olympus XQ10 fiberoptic endoscope (Olympus Corp., New Hyde Park, NY). Anaesthesia was induced with intravenous 20 mg/kg thiopentone sodium (Intraval Sodium, May & Baker, Rhone Merieux, Harlow, UK), the animals intubated with 9.5-10 mm cuffed endotracheal tubes and placed in sternal recumbency. When required, an additional intravenous steroid anaesthetic was employed (Saffan; Pitman-Moore Ltd, Cheshire, UK). Topical anaesthesia of the trachea and major bronchi (5 ml of 2% Xylocaine, Astra, King's Langley, UK) was used to minimise coughing during bronchoscopy (Fig. 2.1).

The 7 mm diameter endoscope was introduced through the endotracheal tube until it wedged in a selected lobar bronchus of the diaphragmatic lobe. 200 ml of sterile physiological saline solution (Aquapharm, Animal Care Ltd, Dunnington, UK) were instilled in 50 ml aliquots via the biopsy channel of the endoscope into the occluded bronchus. BALF was then aspirated gently after each infusion, either using a manual pump (Mityvac pump-3, Arnolds Veterinary Products, Shrewsbury, UK) or 50 ml syringes. The recovered BALF (Fig. 2.2) was pooled in precooled plastic containers and maintained on ice until processed, always within 20 min of collection. Instillation and recovery of the lavage fluid took less than 90 s. The fiberoptic endoscope was always disinfected after use by soaking in a glutaraldehyde solution

Figure 2.1. Performance of bronchoalveolar lavage (BAL) technique in the anaesthetized sheep.

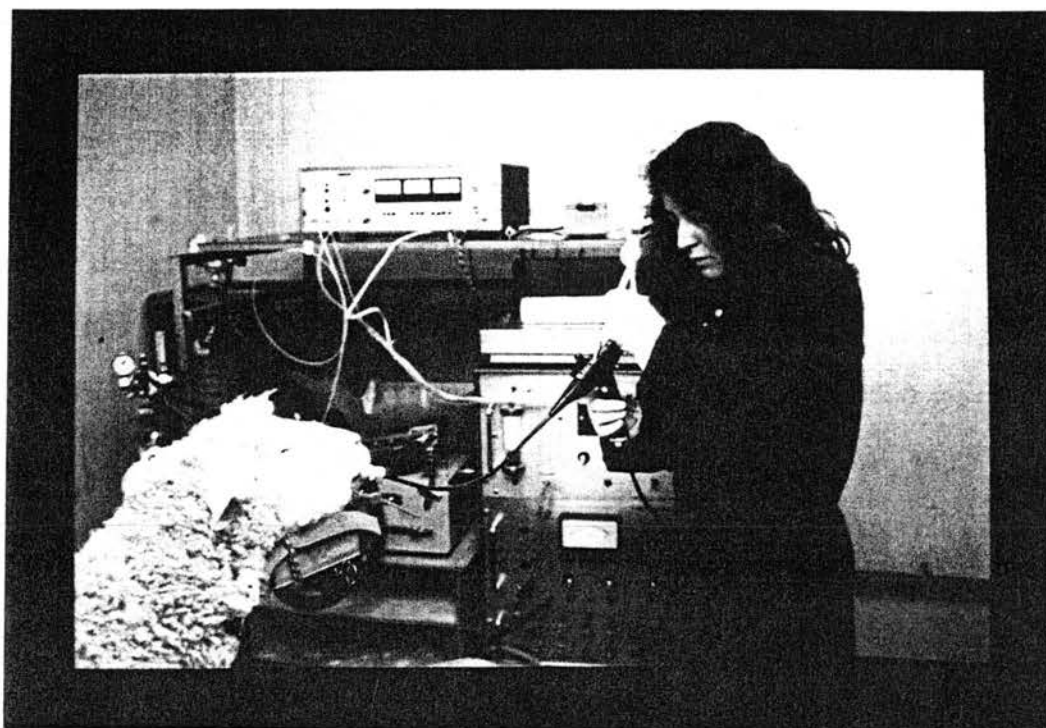


Figure 2.2. Bronchoalveolar lavage fluid (BALF) recovered from a sheep by BAL procedure.



(Cidex, Surgikos, Johnson & Johnson Medical Inc., Arlington, Texas, USA) for at least 30 min.

2.5. PROCESSING OF BALF

BALF was filtered through a loose cotton gauze to remove mucus and the volume recorded. BALF was then centrifuged at 800 g for 10 min at 4°C in a GS-6R centrifuge (Beckman, Palo Alto, California, USA) to sediment cellular material, and the supernatant removed and stored at -20°C. The cell pellet was resuspended in 5 ml of sterile PBS and a total BALF count was performed using a System 9000 automated cell counter (Serono, Baker Diagnostics, Allentown, Pennsylvania, USA). Viability of the BALF cells, as determined by 0.4% trypan blue dye exclusion (Appendix III.6), was always greater than 90%.

2.6. PREPARATION OF CYTOCENTRIFUGE PREPARATIONS

Cytocentrifuge or cytospin preparations were made using a cytocentrifuge (Shandon Southern Instruments Ltd, Cheshire, UK). 50 µl of the BALF cell suspension in PBS at 10^6 cells/ml were centrifuged at 800 g for 7 min. Preparations were then air dried and stained in Leishman's solution (Appendix III.5), or were fixed using a water soluble aerosol fixative (Smear fix, Vale Laboratories, London) and stained with toluidine blue (Appendix III.4).

2.7. DIFFERENTIAL LEUKOCYTE COUNT

Differential counts of 300 BALF cells were performed on Leishman's stained preparations. Absolute BALF cell counts were determined for each cell type by multiplying the cell ratio (%) by the total BALF cell count/100.

2.8. PERIPHERAL BLOOD (PB) COLLECTION

PB samples were collected by jugular venepuncture into evacuated tubes containing potassium ethylene diamine tetra acetic acid (EDTA) (Becton Dickinson, Rutherford, USA).

2.9. SEPARATION OF PERIPHERAL BLOOD LEUKOCYTES

For flow cytometry, peripheral blood leukocytes were harvested from PB following removal of erythrocytes by tris-ammonium chloride lysis ($\text{Tris-NH}_4\text{Cl}$) (Mishell and Shiigi, 1980) (Appendix III.2). A total leukocyte count was performed using a System 9000 automated cell counter (Serono, Baker Diagnostics, Allentown, Pennsylvania, USA).

2.10. IMMUNOFLOUORESCENT LABELLING TECHNIQUE

2.10.1 Single colour flow cytometry

Minor modifications of the methods of Mackay and others (1988b) were adopted in this study. BALF and PB cell suspensions were labelled using an indirect immunofluorescence method. Aliquots of 1.5×10^5 BALF or peripheral blood leukocyte cells were transferred into round-bottomed mini-test tubes (Sterilin, Hounslow, UK), centrifuged at 800 g for 3 min, the cell pellet resuspended in 50 μl of primary MAb or control reagent and incubated at 4°C for 45 min.

The cells were then washed three times in 750 μl of PBA buffer (Appendix II.1) and incubated in 50 μl of fluorescein isothiocyanate conjugated (FITC) F(ab')_2 fragment of rabbit immunoglobulins to mouse IgG (Dako, Copenhagen, Denmark), diluted 1:100 in PBA for 30 min at 4°C in the dark. Prior to use, the diluted FITC-conjugate was centrifuged at 11500 g for 10 min to remove particulate debris.

Following three further washes in PBA buffer, the cells were resuspended in 100 μl PBS containing 1% w/v paraformaldehyde (BDH, Poole, UK) (Appendix II.2)

and maintained in the dark at 4°C to minimise the capping or shedding of surface proteins (Lanier and Warner, 1981) pending analysis, which was always within 24 h of labelling. The control samples were treated similarly except that either the primary MAb was omitted or they were incubated with an irrelevant MAb, namely VPM53, which labels an epitope on *Ileobacter* (McOrist, 1987). The irrelevant MAb was used to establish background fluorescence level.

MAbs and conjugates were titrated to determine their optimal working dilutions (Fig. 2.3). Sheep efferent lymph lymphocytes were stained for single-colour flow cytometric analysis with MAbs VPM37 (Dutia *et al*, 1990a; 1990b), VPM41 (Dutia *et al*, 1990a; 1990b), a pan MHC Class II reagent (VPM36+VPM38) (Dutia *et al*, 1990a; 1990b), VPM8 (Jones, 1988), LFA-1 (Mackay *et al*, 1990) and LFA-3 (Hunig *et al*, 1986), in the dilutions: undiluted, 1/10, 1/20, 1/50 and 1/100. The optimal working dilution of each of the MAbs employed was that which gave the most intense fluorescence (according either to the percentage of positively staining cells or to the mean channel number of the fluorescence intensity) and also the clearest separation between the positive and negative populations. This experiment demonstrated that optimal staining was obtained using all the MAbs as undiluted tissue culture supernatants.

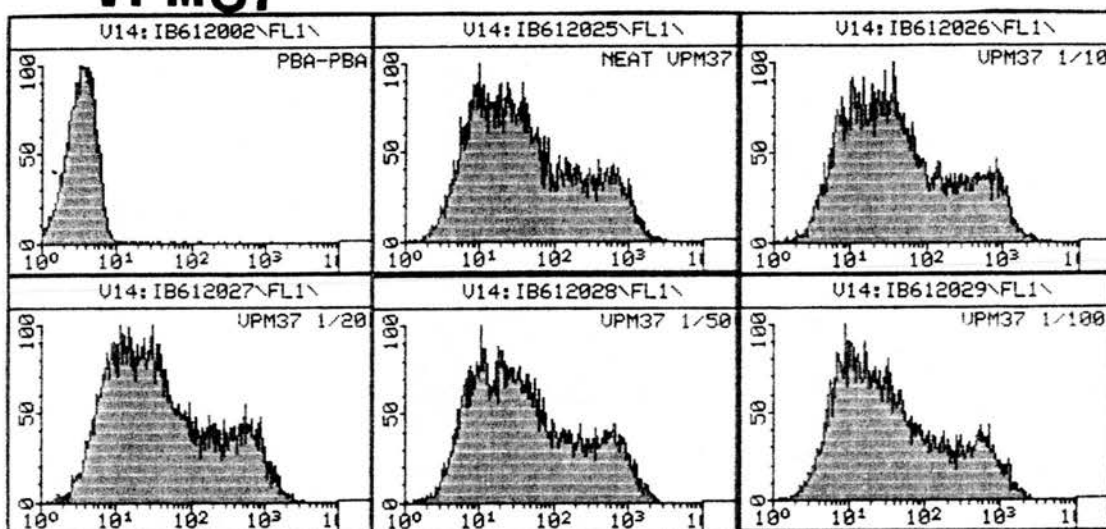
To establish the optimal working dilution of the FITC conjugate, different dilutions of FITC were tested against the undiluted MAbs. The working dilution 1:100 was selected as it gave the strongest specific fluorescence with the lowest background staining of the negative control.

2.10.2 Dual colour flow cytometry

A modification of the sandwich staining procedure described by Pechhold and Kabelitz (1992) was employed. It is a rapid staining procedure that only requires two

Figure 2.3. Titration of MAb. Fluorescence histograms of PBL stained with MAb VPM37, VPM41, anti-Pan Class II (VPM36 + VPM38), LFA-1, LFA-3 and VPM8, used as neat supernatant, and in the following dilutions: 1/10, 1/20, 1/50 and 1/100 are shown. As negative controls, unstained cells were employed (PBA-PBA). Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and linear cell frequency is given on the ordinate.

VPM37



VPM41

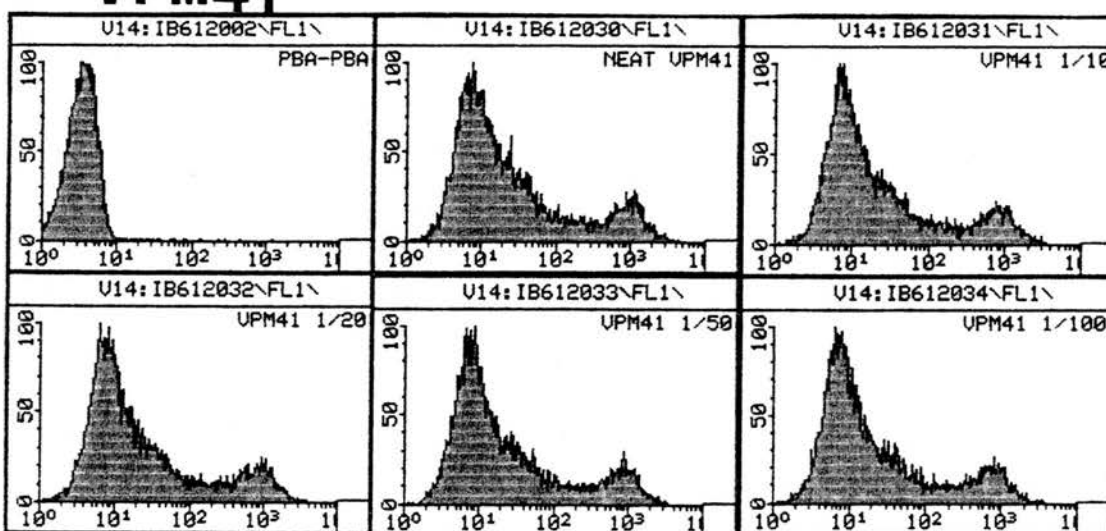
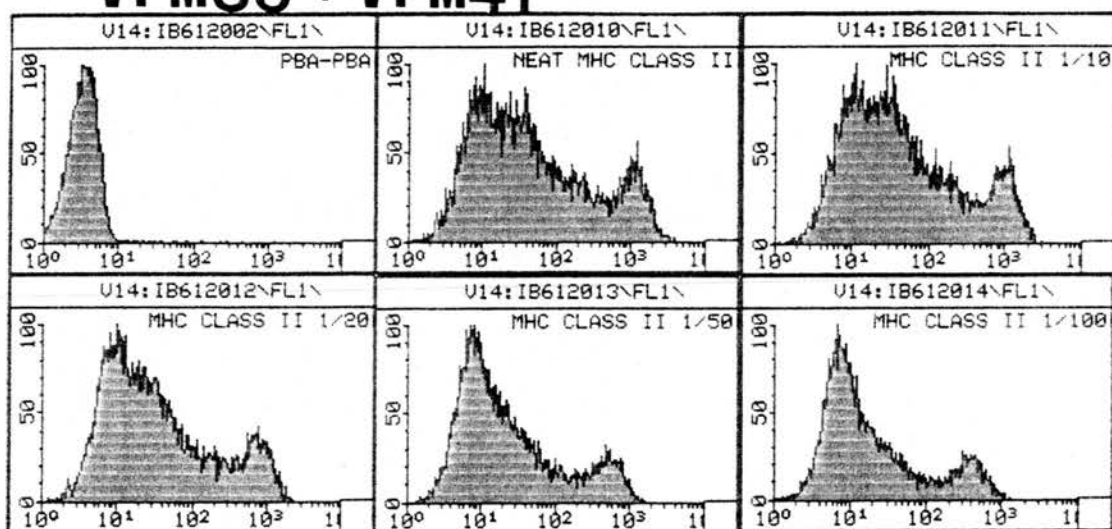


Figure 2.3. Titration of MAb (Continued)

VPM36 + VPM41



LFA-1

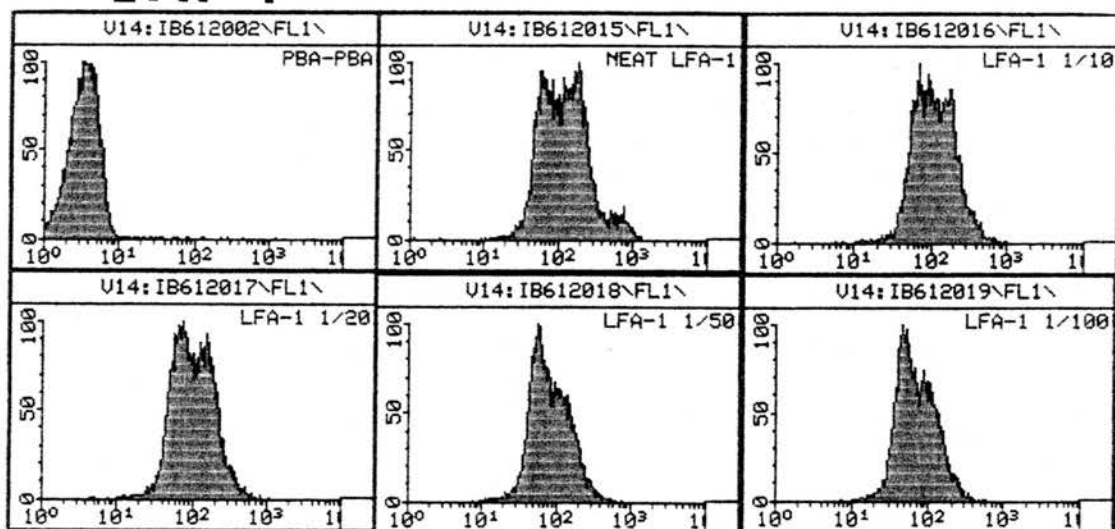
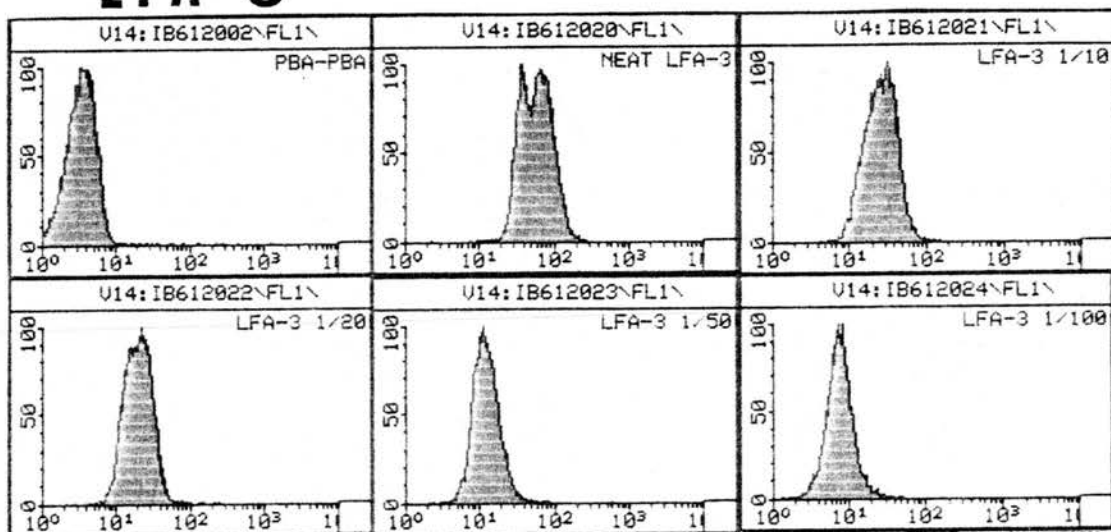
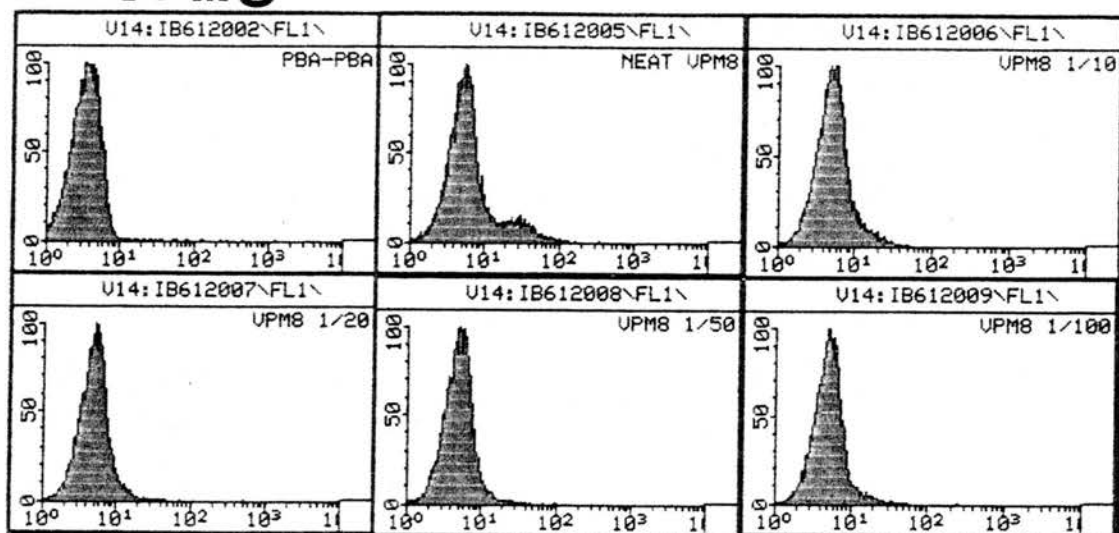


Figure 2.3. Titration of MAb (Continued)

LFA-3



VPM8



incubation steps; firstly, a simultaneous addition of unconjugated and biotinylated MAbs and secondly, the addition of fluorochrome-labelled avidin/streptavidin followed by fluorochrome-labelled Ig.

Essentially, 50 μ l of the BALF or PB cell suspension containing $1.5-2 \times 10^5$ cells/ml were incubated for 30 min at room temperature with combinations of biotinylated (25 μ l) and unconjugated (25 μ l) MAbs. The biotinylated and unconjugated MAbs employed were of different isotypes. Cells were then washed twice in 750 μ l of PBA and 12.5 μ l of 1:200 streptavidin-phycoerythrin (Amersham International, Amersham, UK) was added (for analysis on the FL2 channel which measures red fluorescence). After 5 min, 12.5 μ l of 1:250 isotype specific FITC-conjugated rabbit antimouse IgG₁ (The Binding Site, Birmingham, UK) or 1:500 FITC-conjugated rabbit anti-rat IgG_{2b} (Serotec, Kidlington, UK), depending on the species of origin of the primary MAb, were added (for analysis on the FL1 channel which measures green fluorescence) and incubated for 30 min at 4°C. The cells were again washed with PBA, resuspended in 250 μ l of PBA and kept at 4°C in the dark pending analysis. As analysis was always performed within 4 h, fixation of the cells in a paraformaldehyde solution was not considered necessary. As negative controls, combinations of the biotinylated MAbs plus normal mouse serum (1:1000) (Sigma, Poole, UK) and normal rat serum (1:500) (Sigma, Poole, UK) were included to determine respectively the extent of fluorescent antimouse Ig and anti-rat Ig antibodies binding once avidin has reacted with the biotinylated MAb.

2.11. FLOW CYTOMETRIC ANALYSES

Flow cytometric analyses were carried out on a fluorescence-activated cell analyser, (FACSCAN; Becton-Dickinson, Mountain View, CA, USA) with a 488 nm argon laser. FITC (520nm) emission was detected with the photomultiplier tube voltage set at 555 mV. The Consort 30 Version F programme was used in the

acquisition and storage of the flow cytometric data, while analyses were performed using either Consort 30 or Lysis.

Separate live gates for lymphocytes and for macrophages were set for each BALF and peripheral blood leukocyte sample, based on correlated forward light scatter (FSC) and side light scatter (SSC) parameters. Dead cells were excluded on the basis of their size, indicated by their FSC. A minimum of 5000, and in most cases 10000, gated cells was analysed.

2.12. LYMPHOCYTE CULTURE

2.12.1 Preparation of peripheral blood leukocytes for culture

Hypotonic lysis with tris-ammonium chloride was performed. 10 ml of jugular blood were collected into heparinised vacutainers (Becton-Dickinson, Rutherford, USA), mixed with 35 ml of prewarmed (37°C) tris-ammonium chloride and allowed to stand at room temperature for 3 min for complete lysis of the red blood cells to occur. Then, blood was centrifuged at 800 g in a Megafuge 1.0 (Heraeus, Sepatech, Essex, UK) for 15 min at 4°C and the supernatant discarded. The cell pellet was resuspended in RPMI 1640 'complete culture medium' (Appendix II.5) and washed three times in RPMI 1640 'wash culture medium' (Appendix II.6). Finally, leukocytes from the cell pellet were manually counted using a haemocytometer (Improved Neubauer, Hawksley and Sons, London, UK) and adjusted to the working concentration of 2×10^5 cells/ml in RPMI 1640 'complete culture medium'.

2.12.2 Preparation of BALF lymphocytes for culture

BALF was centrifuged at 800 g for 10 min in a Megafuge 1.0 (Heraeus, Sepatech, Essex, UK) at room temperature and the cell pellet resuspended in 10 ml of RPMI 1640 'complete culture medium' (Appendix II.5). For removal of alveolar macrophages by adherence, BALF was incubated for 90 min at 37°C in a humidified

atmosphere of 5% CO₂ in air using a T-162 (162 cm²) tissue culture flask (Costar, Cambridge, UK) which was kept in a horizontal position, with the cap loosely fitting. After the 90 min incubation, the flask walls were gently flushed with RPMI 1640 'complete culture medium' using a pipette; non-adherent cells were removed and centrifuged at 800 g for 7 min. Leukocytes from the cell pellet were manually counted using a haemocytometer (Improved Neubauer, Hawksley and Sons, London, UK) and adjusted to the working concentration of 2×10^5 cells/ml in RPMI 1640 'complete culture medium'.

2.12.3 Mitogen

A stock solution of concanavalin A (Con A) (Sigma, Poole, UK) at a concentration of 1 mg/ml was prepared in RPMI 1640 'supplemented culture medium' (Appendix II.4), aliquoted and stored at -70°C. The same batch of the mitogen was used throughout the study.

2.12.4 Optimisation of culture system conditions

2.12.4.1 Comparison of peripheral blood lymphocytes (PBL) separation techniques

To determine which lymphocyte separation technique was better for this study, separations by density gradient centrifugation over Lymphoprep (Appendix III.1) and tris-ammonium chloride hypotonic lysis (Appendix III.2) were compared. Leukocytes isolated by each method were stained for CD4, CD8, $\gamma\delta$ T cells, CD5, VPM8 and IL-2R using the appropriate MAbs and then analysed by flow cytometry.

Analyses revealed that when using Lymphoprep a considerable proportion of T-lymphocytes in all the subsets and also lymphocytes expressing IL-2R were lost in comparison to those separated by hypotonic red blood cell lysis with tris-ammonium chloride. However, more B cells were obtained with the density gradient centrifugation over Lymphoprep (Figs. 2.4 and 2.5). In view of these results, and since

Figure 2.4. Light scatter profiles of a PB sample from a control animal subjected to two different lymphocyte separation techniques, namely density gradient centrifugation over Lymphoprep (A) and tris-ammonium chloride buffer lysis (B). More granulocytes (GRA) are present after tris-ammonium chloride lysis.

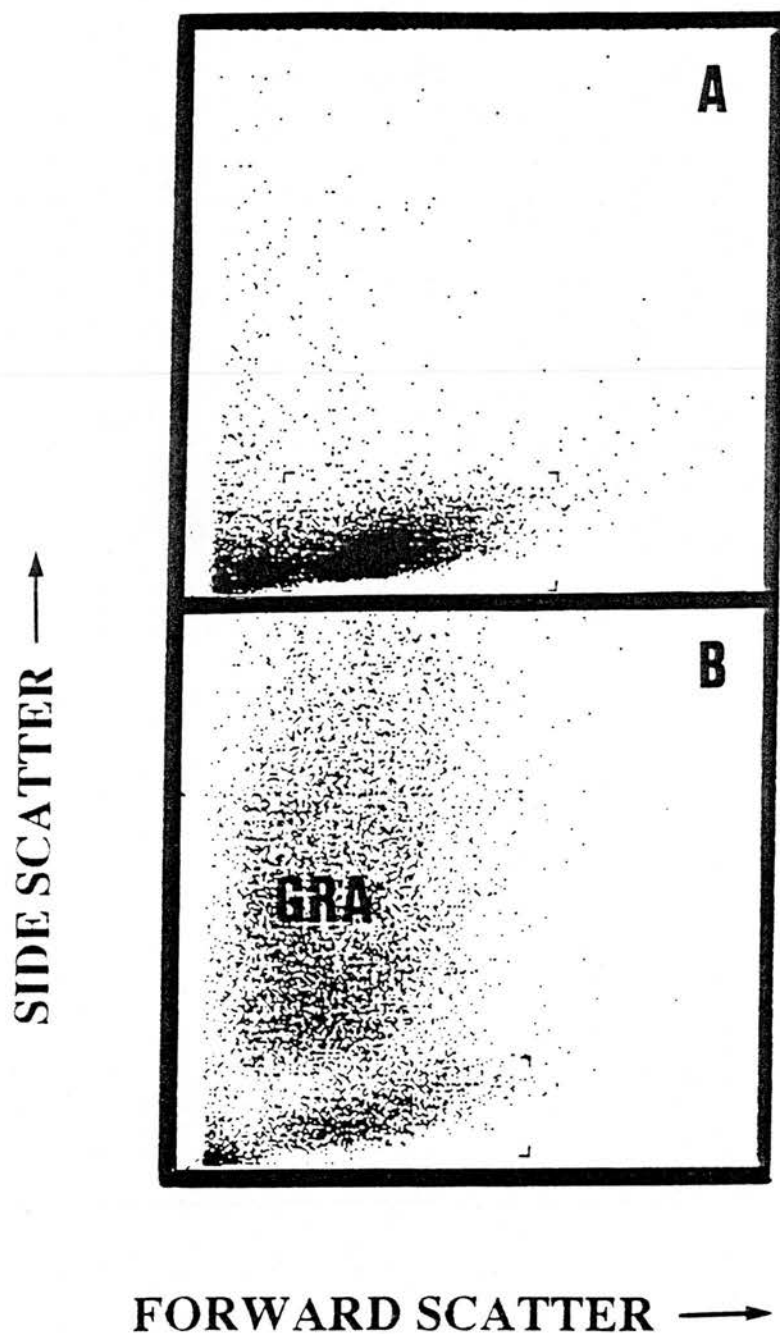
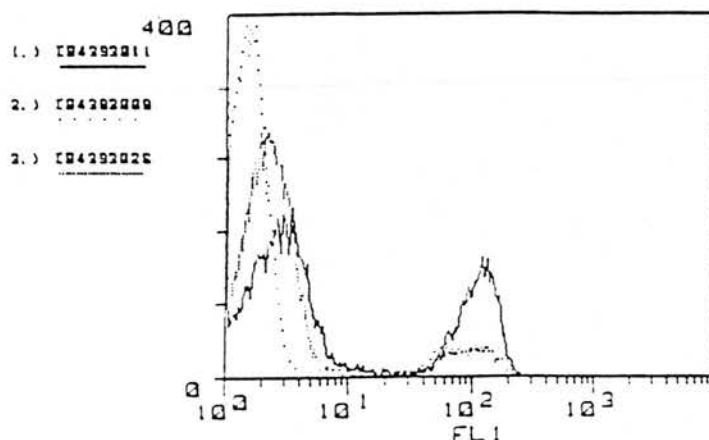


Figure 2.5. Comparison of cell separation techniques in control sheep 96. PBL were separated by either hypotonic lysis of RBC using Tris-ammonium chloride (Tris-NH₄Cl), or by density gradient centrifugation over Lymphoprep, and stained for flow cytometry using 17D.13, SBU-T8, 86D, ST-1, VPM8 and IL-111A, in order to assess the proportion of CD4⁺, CD8⁺, $\gamma\delta$, CD5⁺, B cells and IL-2R stained by each MAb within the lymphocytic gate. Results are expressed as percentages of positively stained cells by reference to a negative control.

a() = cells separated by Tris-NH₄Cl
b(....) = cells separated by Lymphoprep
c(....) = negative control

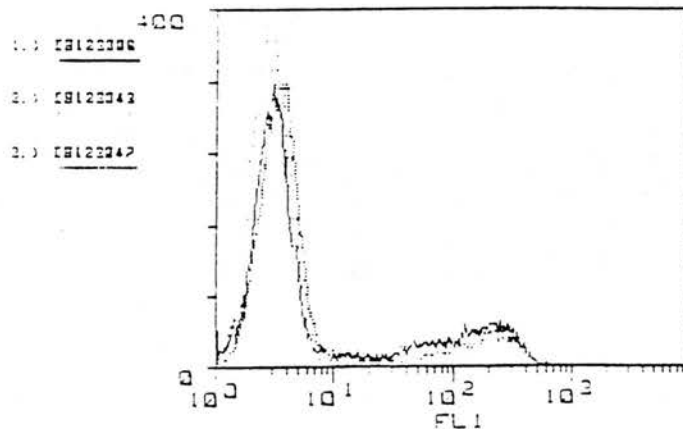
1. CD4⁺ T cells

(a) Tris-NH₄Cl = 31.43%
(b) Lymphoprep = 14.16%

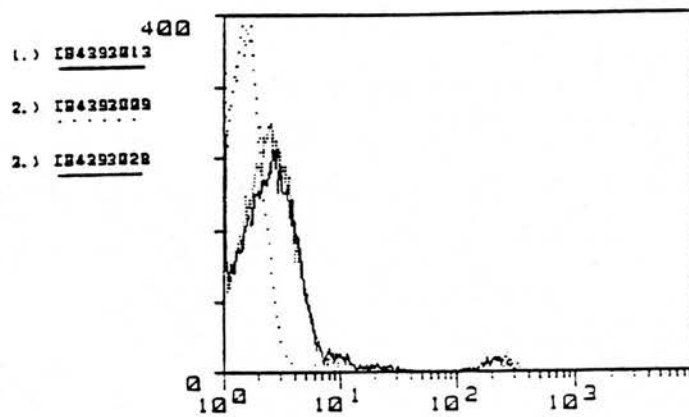


2. CD8⁺ T cells

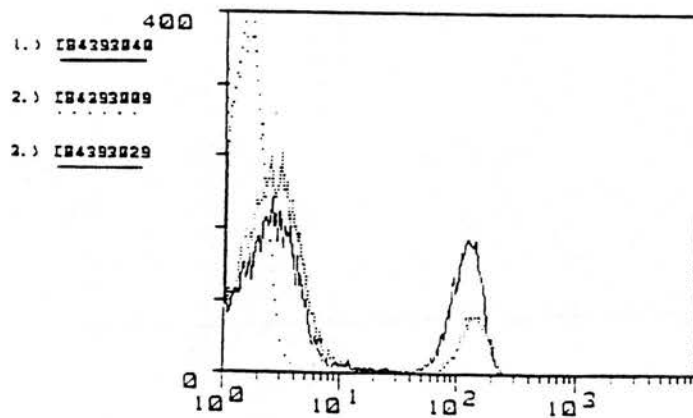
(a) Tris-NH₄Cl = 24.34%
(b) Lymphoprep = 17.01%



3. $\gamma\delta^+$ T cells
 (a) Tris-NH₄Cl = 6.74%
 (b) Lymphoprep = 5.46%

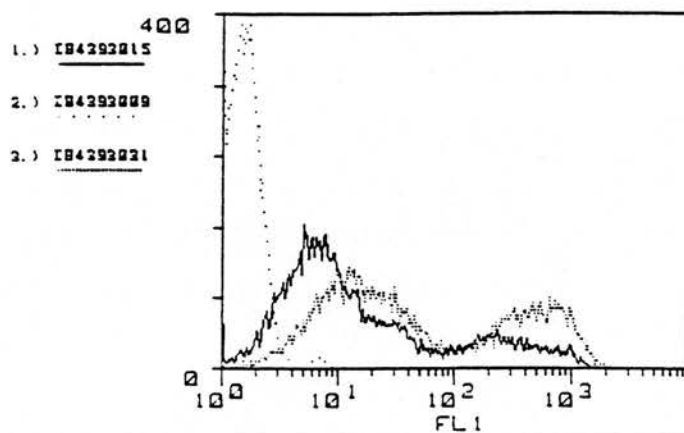


4. CD5⁺ T cells
 (a) Tris-NH₄Cl = 30.4%
 (b) Lymphoprep = 14.51%



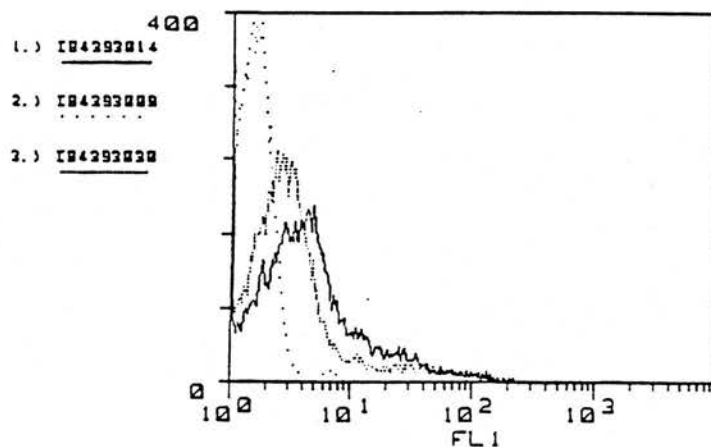
5. B cells

- (a) Tris-NH₄Cl = 21.92%
(b) Lymphoprep = 36.67%



6. IL-2R⁺ cells

- (a) Tris-NH₄Cl = 17.98%
(b) Lymphoprep = 11.75%

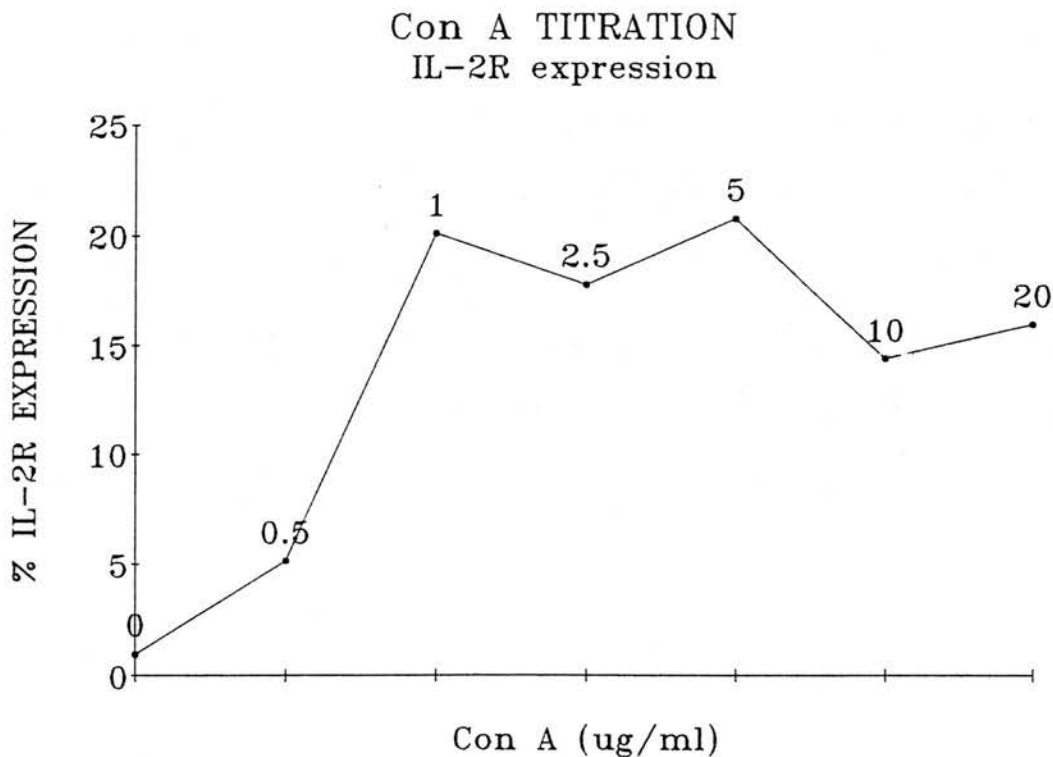


the main purpose of this study was the culture of T-lymphocytes, tris-ammonium chloride hypotonic lysis was the method adopted and employed for subsequent cell separation.

2.12.4.2 *Effect of mitogen concentration*

A range of concentrations of Con A was titrated against a fixed peripheral blood leukocyte concentration of 2×10^5 cells/ml, obtained by tris-ammonium chloride hypotonic lysis, over a 72 h culture period. Duplicate controls were set up for each Con A concentration and also a duplicate control of the cell suspension without mitogen was prepared. The Con A concentrations tested were: 0.5, 1, 2.5, 5, 10 and 20 $\mu\text{g/ml}$. The highest percentage of IL-2R expression was obtained with 1-5 $\mu\text{g/ml}$ of Con A, thus 5 $\mu\text{g/ml}$ was the mitogen concentration adopted throughout the experiment. Results are shown in Fig. 2.6.

Figure 2.6. Con A titration. 2×10^5 peripheral blood leukocytes/ml were cultured in the absence of Con A and with different Con A concentrations: 0.5, 1, 2.5, 5, 10 and 20 $\mu\text{g/ml}$, and the percentage of IL-2R expression 72 h after culture determined.



2.12.5 Culture and stimulation of BALF and PB cells

BALF and PB cell suspensions were cultured with 5 µg/ml Con A (Sigma, Poole, UK) in sterile 24-flat bottomed well plates (Costar, Cambridge, UK). Cultures to which no mitogen was added were employed as controls. All cultures were set in duplicates and incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for 3 days. Microplates were then centrifuged at 800 g for 7 min, supernatants stored at -20°C for further immunological assays and cell pellets were recovered by flushing the wells with RPMI 1640 'complete culture medium'.

2.12.6 Flow cytometric analyses in cultured lymphocytes

Dual-colour flow cytometric analyses were performed on cultured lymphocytes. The modified sandwich staining procedure described in section 2.10.2 was employed (Pechhold and Kabelitz, 1992). Flow cytometric analyses were carried out as previously described.

2.13. STATISTICAL ANALYSES

Cell counts, cell ratios, BALF volumes and flow cytometric data were non-normally distributed (Merchant *et al*, 1992; Schwartz *et al*, 1993). Hence, all the paired and unpaired sample comparisons were made using the non-parametric Wilcoxon Rank Test or the Mann-Whitney Test respectively, with a significance level of 95%. Statistical analyses were performed using Minitab (Minitab Inc., Pennsylvania, USA).

2.14. VIRUS INOCULUM FOR EXPERIMENTAL MVV INFECTION

The virus isolate employed for the experimental MVV infection is a British isolate of MVV, designated EV1 (Sargan *et al*, 1991). This virus was first isolated by cocultivation of PBMCs from a sheep with an acute maedi-like disease (displaying symptoms of arthritis and pneumonia) with an ovine skin cell line (derived by explant

and cultivation of a skin biopsy from an uninfected sheep) until syncytia appeared (approximately 12 days). Extracellular virus was passaged twice more through these cells (m.o.i. < 0.1 TCID₅₀/cell) and harvested at 12 days post-infection. Cultured fluid was finally harvested, divided into 5 ml aliquots and stored at -70°C.

2.15. TGF- β GROWTH INHIBITION ASSAY

TGF- β was quantified by its ability to inhibit epithelial cell proliferation. Plasma and BALF supernatant samples were assayed for TGF- β activity using a mink lung epithelial cell line, MV.1.Lu (ECACC, Porton Down, Salisbury, Wiltshire, UK). This method is based on the procedures of Tucker and others (1984) and Ikeda and others (1987).

2.15.1 Preparation of mink lung epithelial cells

MV.1.Lu mink lung epithelial cells were maintained in the high glucose formulation of DMEM medium (Gibco Ltd., Paisley, Scotland) supplemented with 10% heat-inactivated foetal calf serum (Ato Tek, Ltd., Glasbury, Hereford, UK) (Appendix II.7). Cells were kept at 37°C in 10% CO₂ and passaged at a seed density of 5×10^5 cells/T-75 (75 cm²) tissue culture flask (Costar, Cambridge, UK) at 3-day intervals. Subconfluent cells were used to initiate the growth inhibition assay.

To detach cells from the flask surface, 5 ml of versene (BDH, Poole, UK) (Appendix II.8) were added, incubated for approximately 2 min and discarded. A combination of trypsin (DIFCO, Detroit, Michigan, USA) and versene (BDH, Poole, UK) (1:4) (Appendix II.9), sufficient to cover the cells, was then added and incubated for 5 min. Recovered cells were resuspended in 4 ml of 'supplemented DMEM medium' (Appendix II.7) and centrifuged for 10 min at 800 g. Supernatant was discarded, and the cell pellet resuspended in 3 ml of 'supplemented DMEM medium'. A cell count was performed using a haemocytometer and the cell concentration was adjusted to $3-5 \times 10^4$ cells/well.

2.15.2 Acidifying procedure

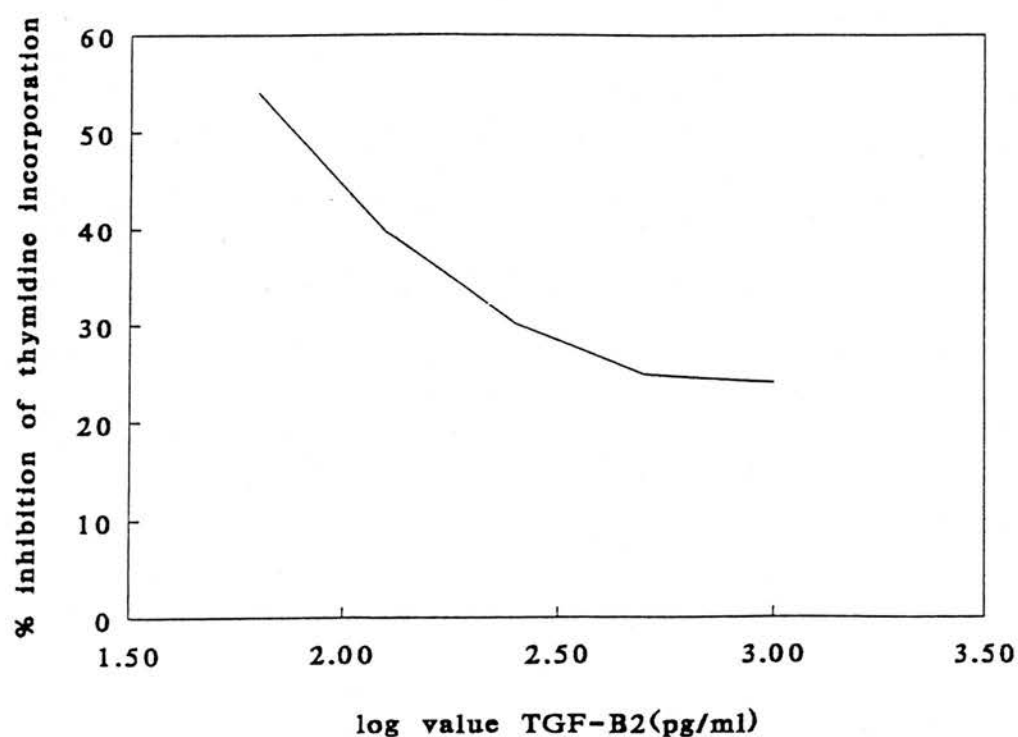
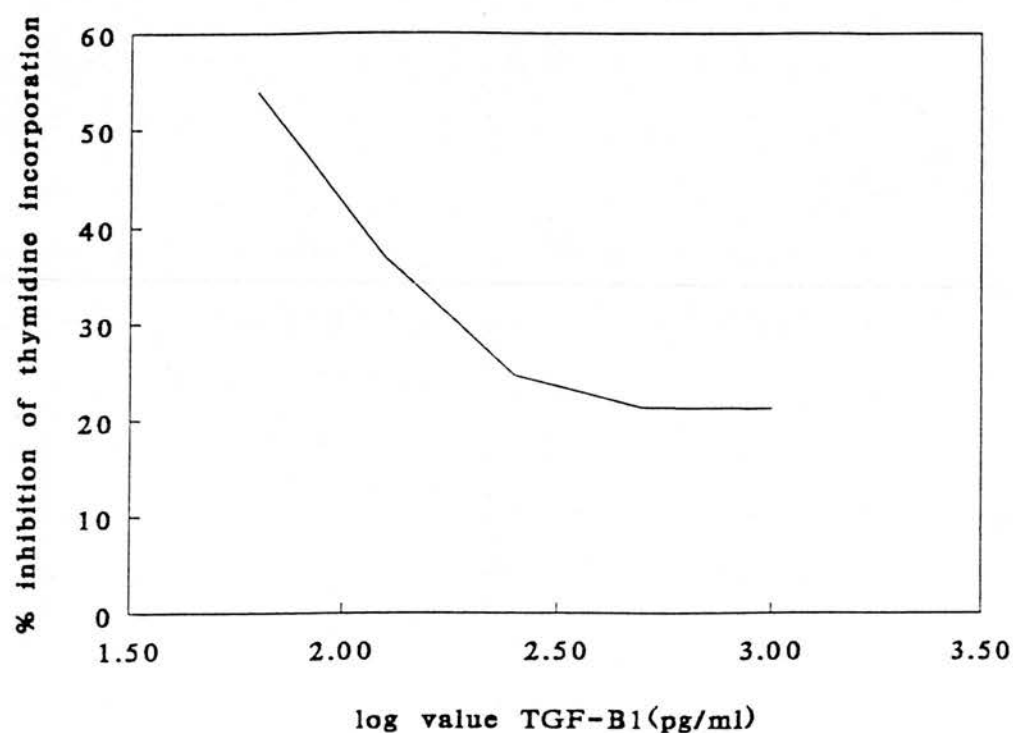
Since TGF- β is normally secreted in a latent form, BALF supernatants and plasma samples had to be transiently acid-activated. Acidification was accomplished by addition of 3 μ l of 5 M HCl to 200 μ l of the samples to reach a pH of 1.5 to 3, and left at room temperature for 30 min. Samples were then neutralised with 10.6 μ l of a 1.4 M NaOH dilution in 0.7 M Hepes to a pH of 7.4. Finally, 600 μ l of 'supplemented DMEM medium' were added.

2.15.3 Assay for TGF- β

Cells were seeded at $3\text{--}5 \times 10^4$ cells/well in a volume of 100 μ l 'supplemented DMEM medium' in 96-well flat bottomed microtitre plates. After 1 h, 100 μ l of twofold dilutions of acidified plasma and BALF supernatant samples or 100 μ l of serial dilutions of a TGF- β_1 , and occasionally a TGF- β_2 , standard preparation (NIBSC, Hertfordshire, UK) were added. 22 h later the medium was removed and cells were pulsed for 2 h with ^3H -TdR (American International, Amersham, UK) (specific activity of 1 μ Ci/well) at 37°C. At the end of the pulsing period, cells were washed twice, first with 200 μ l of trypsin/versene, and then with 100 μ l of trypsin, and left at 37°C in 10% CO₂ for 30 min, to induce cell detachment. Cells were then harvested onto Wallac glass fiber filter mats (Wallac Oy, Turku, Finland) using a Harvester 96 (Tomtec, Orange, Conn, USA), and finally radioactivity was determined in a LKB Wallac 1218 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland).

Each sample was assayed in triplicate. Concentrations of TGF- β were expressed in ng/ml based on a standard curve that was generated with each set of assays by using either human recombinant TGF- β_1 or TGF- β_2 (NIBSC, Hertfordshire, UK) (Fig. 2.7).

Fig. 2.7. TGF- β dose response curve. MV.1.Lu mink lung epithelial cells were plated at a density of 3.5×10^4 cells/well and treated with 100 μ l of serial dilutions of a TGF- β_1 or TGF- β_2 for 22 h. Conditioned media were then collected and cells were pulsed for 2 h with ^3H -TdR at 37°C. TGF- β activity was expressed as percent inhibition of cell proliferation by the indicated dilutions of TGF- β_1 or TGF- β_2 . The results were from triplicate determinations at each dose of TGF- β .



2.15.4 Antibody neutralization

To block TGF- β activity, BALF supernatant and plasma were incubated with chicken monospecific polyclonal antibodies to TGF- β . Samples were incubated at 37°C in 10% CO₂ for 1 h with 1:100 dilution of anti-TGF- β ₁ (NIBSC, Hertfordshire, UK), anti-TGF- β ₂ (NIBSC, Hertfordshire, UK) or nonimmune chicken IgG₁ (Sigma, Poole, UK). The samples were then tested in the mink lung epithelial cell inhibition assay as described above.

2.16. PLASMA AND BALF UREA DETERMINATIONS

BALF supernatant urea concentrations were determined using a commercial enzymatic assay kit (Bun Endpoint, Sigma, Poole), employing a modification of the technique of Rennard and others (1986). To minimise bacterial degradation of urea, samples were processed and assayed immediately after thawing to room temperature. Prior to assay, BALF supernatants were centrifuged at 12000 g for 5 min (Maxifuge, Whiteleaf Scientific, Dulverton) to remove precipitated matter which could affect optical density measurements. Reaction mixtures of 1.04 ml freshly prepared reagent and 0.21 ml BALF supernatant or urea standard were gently mixed within the cuvettes (Semimicro, BDH, Poole) and incubated at room temperature for 5 min. The absorbance was determined spectrophotometrically (CE 292, Cecil Instruments, Cambridge) at 340 nm using a 0.5 cm light path. The mean absorbances of duplicate samples were subtracted from the sum of the absorbance of a reagent blank (1.04 ml reagent and 0.21 ml distilled water) and that of a BALF supernatant blank (1.04 ml distilled water and 0.21 ml BALF supernatant) to yield the change in absorbance.

The urea concentration was determined from a standard curve prepared using a glucose/urea nitrogen standard solution (Sigma, Poole, UK).

Plasma urea concentrations were kindly determined by the Clinical Diagnostic Laboratory, R(D) SVS using a Technicon RAXT (Technicon, Dublin).

2.16.1 Determination of PELF TGF- β concentrations

Assuming that urea, being a small freely diffusible molecule, is present in equal concentrations in plasma and in PELF, the concentration of PELF in BALF samples can be determined by the urea dilution technique (Rennard *et al*, 1986).

The plasma: BALF urea ratios were determined for each sample. The products of these ratios and the BALF TGF- β concentration yielded the urea adjusted BALF TGF- β concentration, which was considered to be the PELF TGF- β concentration.

$$[\text{PELF TGF-}\beta] = [\text{BALF TGF-}\beta] \times [\text{PLASMA UREA}] / [\text{BALF UREA}]$$

CHAPTER 3: PHENOTYPIC ANALYSIS OF CELLS IN BRONCHOALVEOLAR LAVAGE FLUID (BALF) AND PERIPHERAL BLOOD (PB) OF MAEDI-VISNA INFECTED SHEEP

3.1. SUMMARY

A phenotypic analysis of the cells present in PB and BALF of maedi-visna virus (MVV) seropositive (n=12) and control sheep (n=13) was performed. Cells were labelled by indirect immunofluorescence using MAbs for leukocyte common antigen (LCA), CD4, CD8, $\gamma\delta$ -TCR, CD5, IgG light chain, three different MHC Class II reagents and the lymphocyte function-associated antigens 1 (LFA-1) and 3 (LFA-3), and analysed by flow cytometry. BALF from MVV-infected sheep had significantly increased percentages of lymphocytes, neutrophils and CD8⁺ lymphocytes and decreased percentages of CD4⁺ and CD5⁺ lymphocytes ($p < 0.05$). The CD4⁺/CD8⁺ ratio was significantly inverted in MVV-infected sheep ($p < 0.001$). The percentages of $\gamma\delta$ T lymphocytes and B lymphocytes did not differ significantly from those of the controls. MVV-infected animals showed increased MHC Class II expression on BALF macrophages and upregulated expression of LFA-1 and LFA-3 on BALF lymphocytes ($p < 0.05$). There were no significant differences in lymphocyte phenotype distributions in PB between MVV-infected sheep and controls. The lymphocyte phenotype distributions of BALF and PB were not significantly correlated. These data show the phenotypic changes occurring in the cellular components of BALF during the course of natural MVV infection in live sheep, and their possible relationship with the pathogenesis of lentiviruses. Other findings that could contribute to a better understanding of the pulmonary immune response during MVV infection are also reported and discussed.

3.2. INTRODUCTION

The mucosal lining of the mammalian respiratory tract contains many components of the immune system, including phagocytes, lymphocytes and immunoglobulins, all of which become involved in immune and inflammatory reactions. By analysing representative samples of pulmonary secretions, therefore, a great deal of information about the nature of respiratory diseases can be obtained (Mair, 1987).

Bronchoalveolar lavage (BAL), performed using a fiberoptic endoscope, has enabled sampling of the cell populations resident within the airways. The cell population harvested by this procedure correlates fairly well with cell populations obtained from fractionated lung preparations (Curtis and Kaltreider, 1989) and transbronchial lung biopsies (Tomichi *et al*, 1989). This, together with the safety and repeatability of the procedure, has made BAL one of the main techniques for the collection of samples from the lower respiratory tract.

The study and characterisation of MAbs to sheep lymphocyte antigens has allowed detailed studies of lymphocyte subpopulations from different ovine tissues such as jejunal Peyer's patches (Hein *et al*, 1989), large intestinal lymphoid follicles (Aleksandersen *et al*, 1990) and mammary gland (Lee *et al*, 1991), facilitating the investigation of cellular aspects of the sheep immune system (Mackay *et al*, 1985; Maddox *et al*, 1985b; Beya and Miyasaka, 1986; Beya *et al*, 1986; Hopkins *et al*, 1986). Some MAbs against ovine macrophages have also been recently developed (Pepin, 1992).

Immunofluorescence labelling with MAbs and flow cytometry are techniques with great relevance as research tools having numerous applications in biology and medicine. They have enabled studies of the density and distribution of cell surface determinants and the identification of functional subpopulations of cells. This

methodology has been applied to the investigation of pulmonary immune responses and has proved to have many advantages over other available immunological techniques. Hence, phenotypic study of the cellular components of BALF has been widely reported in man (Yamada *et al*, 1986), mice (Curtis and Kaltreider, 1989), rabbits (Shellito *et al*, 1981), rats (Thrall and Barton, 1984), pig (Gehrke and Pabst, 1990, Asai *et al*, 1993) and horses (McGorum, 1992; McGorum *et al*, 1993). Phenotypic analyses of ovine lymphocytes and macrophages recovered by BAL have also been reported (Cordier *et al*, 1990; Nash *et al*, 1992; Burrells and Sutherland, 1994).

Phenotypic analyses of BALF cells have proven extremely useful in the investigation of several pulmonary inflammatory conditions including human AIDS (Randall Young *et al*, 1985), asthma (Smith and Deshazo, 1993), pulmonary sarcoidosis (Hunninghake and Crystal, 1981), hypersensitivity pneumonitis (Keller *et al*, 1984), idiopathic pulmonary fibrosis (Ginns *et al*, 1982) and equine chronic obstructive pulmonary disease (McGorum *et al*, 1993).

Although phenotypic analyses of BALF lymphocytes and macrophages in MVV-infection (Cordier *et al*, 1990) had already been performed, the present work can be justified as it differs in several important respects with that of Cordier and others. Simultaneous comparison of PB and BALF parameters were not seen in the previous studies. Furthermore, Cordier and others employed excised lungs obtained from the abattoir for the collection of BALF, while the present study was performed on BALF collected from live animals. Cordier and others (1990) did not determine the serological status of the animals employed, while in the work described here all the infected animals were seropositive to MVV. Besides, both the control and the MVV-infected sheep used in this study were breed-, sex- and age-matched. Finally, except for one common MAb, SBU-T8, all the other MAbs used by Cordier and others

differed from those used in this work. In spite of these differences, Cordier and others' data are useful parameters with which to compare and discuss the present results.

The aims of this study were to characterise the immunoregulatory cells present in PB and BALF from control and naturally MVV-infected animals in a flock of British Texel ewes; to determine whether alterations of these cells occur, and if so, to provide some insight into the possible contribution of these immune cell populations to the pathogenesis of maedi.

3.3. MATERIALS AND METHODS

3.3.1 ANIMALS

Twelve adult Texel ewes (body weight range 40-75 kg), seropositive for MVV by the agar gel immunodiffusion test (AGIDT) and thirteen breed, sex and age matched seronegative controls (body weight range 53-87 kg) were used. Both groups of animals were kept under similar management conditions as described in Chapter 2.

3.3.2 SAMPLE COLLECTION AND PROCESSING

PB and BALF samples were collected and processed as previously described (Chapter 2).

3.3.3 MONOCLONAL ANTIBODIES

The murine MAbs used as primary stage reagents are listed in Table 3.1. As a second stage reagent a fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of rabbit immunoglobulins to mouse Ig (Dako, High Wycombe) was employed at a previously determined optimal dilution of 1:100.

Table 3.1. List of monoclonal antibodies (MAbs) employed for single-colour flow cytometric analyses.

MABs	ISOTYPE	ANTIGEN IDENTIFIED	REFERENCE
LYMPHOCYTE MARKERS			
VPM18	IgG ₁	Ovine CD45R	Hopkins & Dutia, 1990
I7D.13	IgG ₁	Ovine CD4	Mackay <i>et al</i> , 1988a
SBU-T8	IgG _{2a}	Ovine CD8	Maddox <i>et al</i> , 1985b
86D	IgG ₁	Ovine WC2 ($\gamma\delta$ T cells)	Mackay <i>et al</i> , 1989
F10/91/43	IgG ₁	Ovine CD5	Beya <i>et al</i> , 1986
VPM8	IgG ₁	Ovine light chain Ig	Jones, 1988
LYMPHOCYTE AND MACROPHAGE MARKERS			
VPM36+38	IgG ₁	Total ovine MHC Class II α	Dutia <i>et al</i> , 1990a; 1990b
VPM37	IgG ₁	Ovine MHC Class II β (DR)	Dutia <i>et al</i> , 1990a; 1990b
VPM41	IgG ₁	Ovine MHC Class II β (DQ)	Dutia <i>et al</i> , 1990a; 1990b
F10/150/39	IgG ₁	Ovine LFA-1	Mackay <i>et al</i> , 1990
L180/1	IgG ₁	Ovine LFA-3	Hunig <i>et al</i> , 1986
IRRELEVANT NEGATIVE CONTROL			
VPM53	IgG ₁	Ileobacter-like organisms	McOrist <i>et al</i> , 1987

3.3.4 COMPARISON OF ERYTHROCYTE LYSIS TECHNIQUES

When performing flow cytometry on PB lymphocyte subpopulations which have been selected using forward (FSC) and side (SSC) light scatter parameters, it may prove difficult to exclude erythrocytes from the 'analysis gate', particularly if large immature erythrocytes are present. To eliminate this problem, contaminant erythrocytes must be lysed prior to flow cytometric analysis. The success of erythrocyte lysis can then be assessed by determining the percentage of leukocytes present in the gated leukocyte population using flow cytometry following labelling with an antibody against the leukocyte common antigen (LCA).

Two techniques of erythrocyte lysis, namely osmotic shock lysis (Appendix III.3) and tris-ammonium chloride buffer hypotonic lysis (Appendix III.2) were compared. Aliquots of PB from seven control animals were subjected to both erythrocyte lysis techniques and their lymphocyte phenotype distributions determined by flow cytometry following staining for CD4, CD8, $\gamma\delta$ T cells, CD5 and B cells.

3.3.5 IMMUNOFLUORESCENT LABELLING TECHNIQUE

BALF and PB cell suspensions were labelled using the single colour labelling technique described in Chapter 2.

3.3.6 FLOW CYTOMETRIC ANALYSES

Samples were analysed as described in section 2.11. A minimum of 5000, and in most cases, 10000 individual cells from each sample were collected and the analyses were performed by setting separate live gates around the lymphocyte or macrophage populations respectively based on correlated FSC and SSC parameters. FSC and SSC amplifications were linear, while FITC amplification was logarithmic (all 256 channels). FSC and SSC scatter profiles of PB enabled the identification of four different cell populations considered to represent dead cells/erythrocytes,

lymphocytes, monocytes and granulocytes (Fig. 3.1). The scatter profiles of BALF showed three different cell populations: erythrocytes/dead cells, lymphocytes and macrophages/granulocytes.

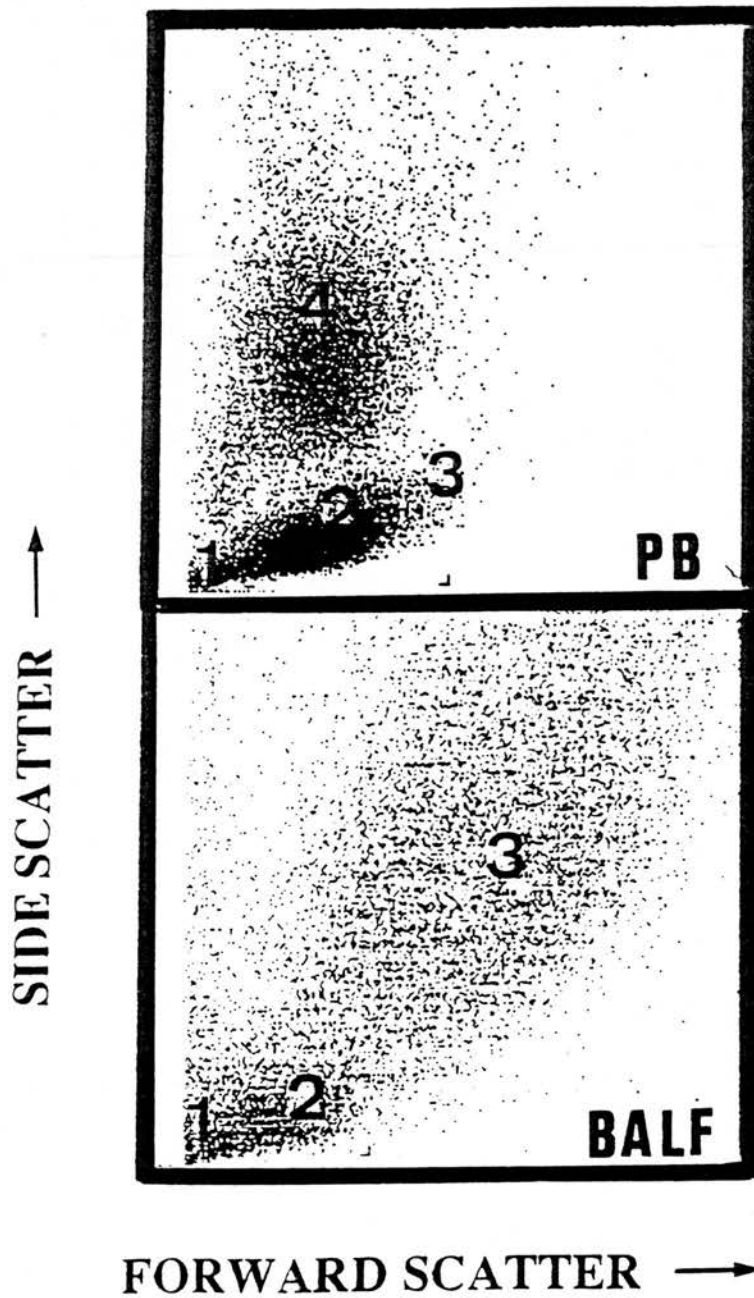
Data were presented as fluorescence profiles with the cell frequency on the ordinate and the log fluorescence intensity on the abscissa. Consort 30 and Lysis software packages (Becton Dickinson Ltd., Los Angeles, California, USA) were used to analyse these data. The results were expressed either as the percentage of cells positively stained or as the relative fluorescence intensity (the 'mode' or 'mean channel number'). The mean channel number of a particular fluorescence profile provides an indication of the intensity of fluorescence either on, or within the cells under examination.

To confirm whether or not the differences found in the expression of some of the markers were due to differences in the size or complexity of the cells, the FSC and SSC measurements on the different cell populations of the MVV-infected animals were compared with those of controls.

3.3.7 STATISTICAL ANALYSES

As data were not normally distributed, paired and unpaired sample comparisons were made using the non-parametric Wilcoxon Rank Test and the Mann-Whitney Test respectively, assuming a significance level of 95%.

Figure 3.1. Light scatter profiles of ovine PB and BALF. Distinct populations of cells have characteristic scatter profiles which can be defined by electronic gates on the flow cytometer. The four identifiable cell populations on the PB profile are labelled as (1) dead cells/erythrocytes, (2) lymphocytes, (3) monocytes and (4) granulocytes. The three identifiable cell populations on the BALF profile are labelled (1) erythrocytes/dead cells, (2) lymphocytes and (3) macrophages/granulocytes.



3.4. RESULTS

3.4.1 CLINICAL STATUS OF THE ANIMALS

MVV-infected sheep had lower body condition than controls, but showed no other clinical signs of respiratory disease. MVV-infected animals had varying degrees of subclinical pulmonary dysfunction as assessed by pulmonary function testing (Collie *et al*, 1993). Clinical and haematological examination did not indicate the presence of any other significant respiratory disease unrelated to MVV infection in either group.

3.4.2 COMPARISON OF ERYTHROCYTE LYSIS TECHNIQUES

Scatter profiles from those PB samples which had been subjected to tris-ammonium chloride hypotonic lysis had larger numbers of cells which had FSC and SCC profiles consistent with those of lymphocytes and granulocytes (Fig. 3.2). There was no statistical difference in the lymphocyte phenotype distribution of PB samples subjected to the two different lysis techniques (Fig. 3.3, Table 3.2). Tris-ammonium chloride was used for subsequent studies.

Figure 3.2. Light scatter profiles of a PB sample from a control animal subjected to two different erythrocyte lysis techniques, namely osmotic shock lysis (A) and tris-ammonium chloride buffer lysis (B). More lymphocytes (LYM) and granulocytes (GRA) are present after tris-ammonium chloride lysis.

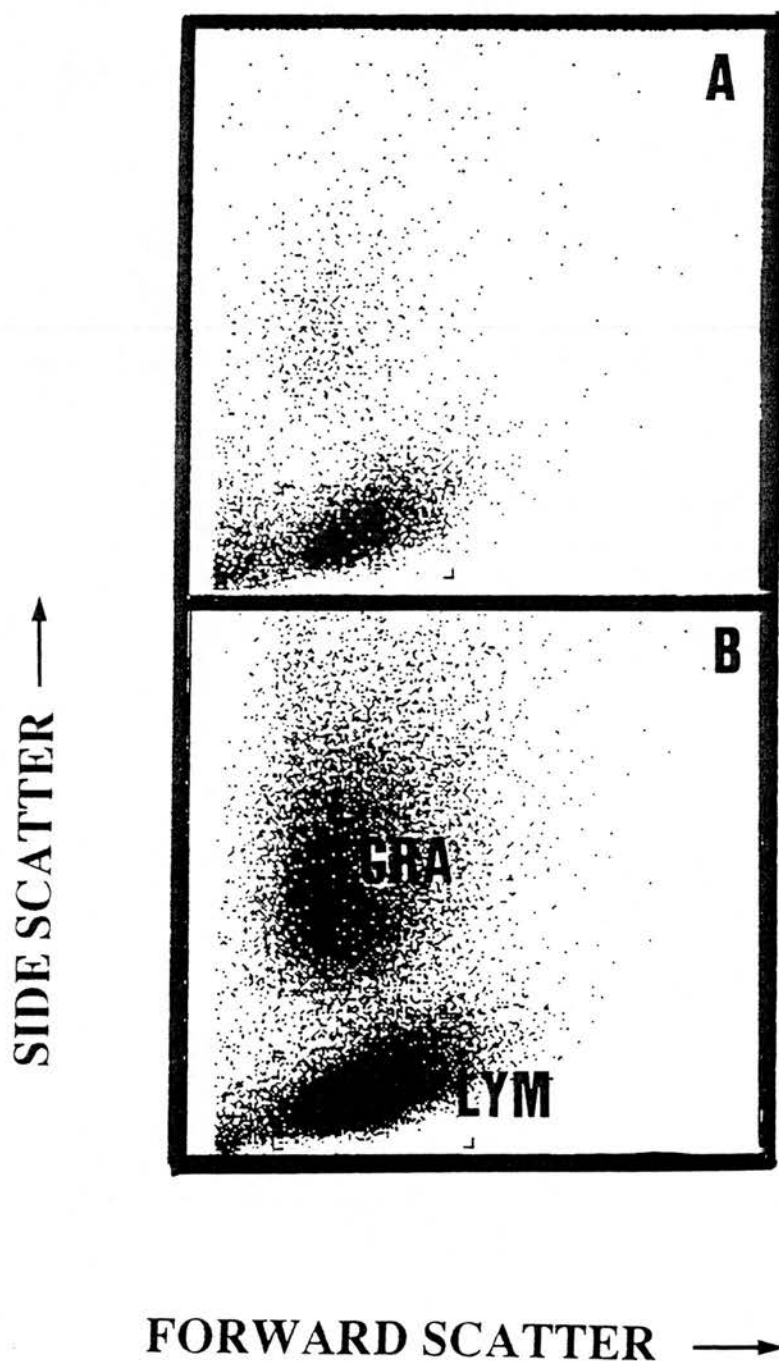


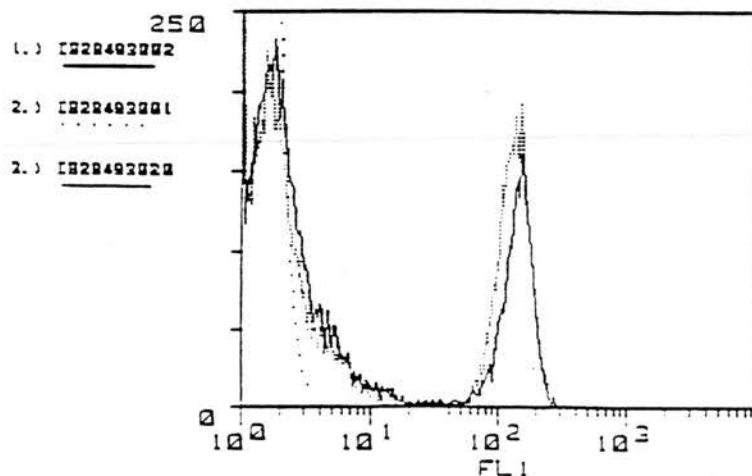
Figure 3.3. Comparison of erythrocyte lysis techniques in control animal 96. Erythrocytes were lysed either by osmotic shock lysis or by Tris-ammonium chloride buffer (NH_4Cl), and PBL were stained for flow cytometry using 17D.13, SBU-T8, 86D, ST-1 and VPM8, in order to assess the proportion of CD4^+ , CD8^+ , $\gamma\delta$, CD5^+ and B cells within the lymphocytic gate. Results are expressed as percentages of positively stained cells by reference to a negative control.

a(—) = cells separated by osmotic shock lysis.
 b(....) = cells separated by Tris- NH_4Cl
 c(....) = negative control

1. CD4^+ T cells

(a) osmotic shock lysis = 36.59%

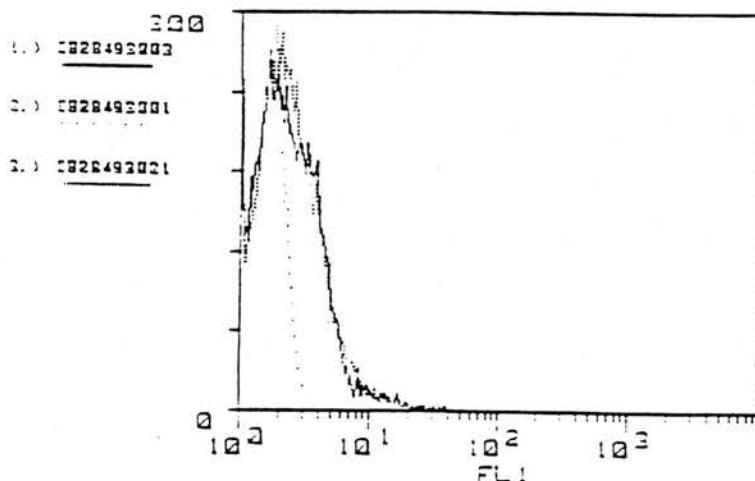
(b) Tris- NH_4Cl = 41.39%



2. CD8^+ T cells

(a) osmotic shock lysis = 16.60%

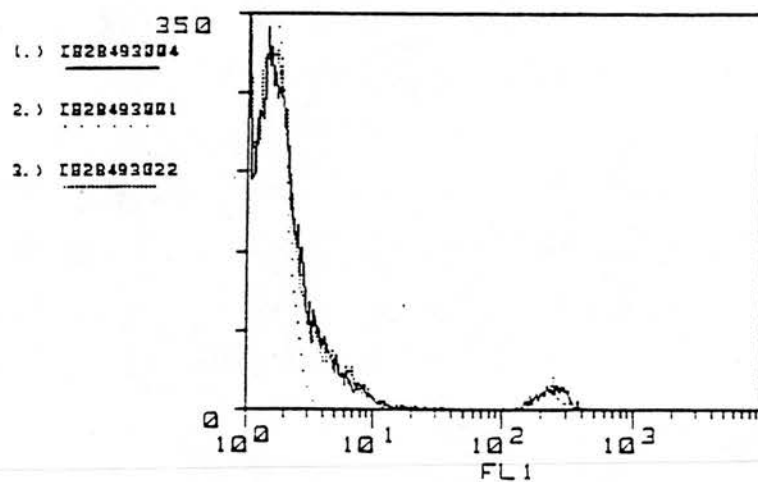
(b) Tris- NH_4Cl = 13.71%



3. $\gamma\delta^+$ T cells

(a) osmotic shock lysis = 14.00%

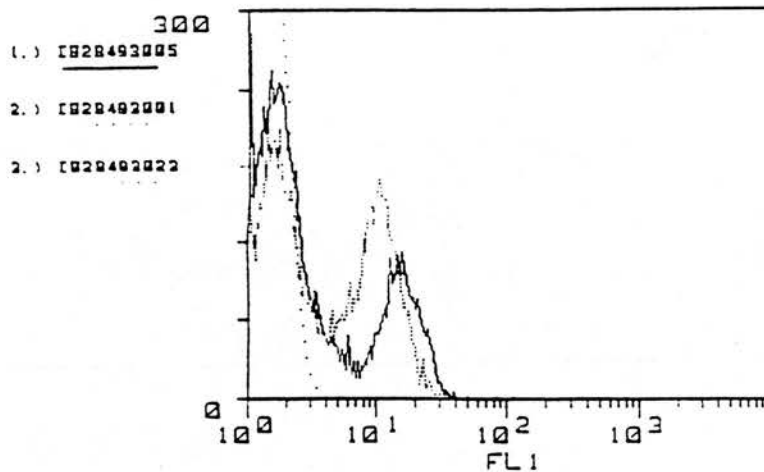
(b) Tris- NH_4Cl = 11.11%



4. CD5⁺ T cells

(a) osmotic shock lysis = 31.86%

(b) Tris- NH_4Cl = 42.93%



5. B cells

(a) osmotic shock lysis = 32.34%

(b) Tris- NH_4Cl = 33.20%

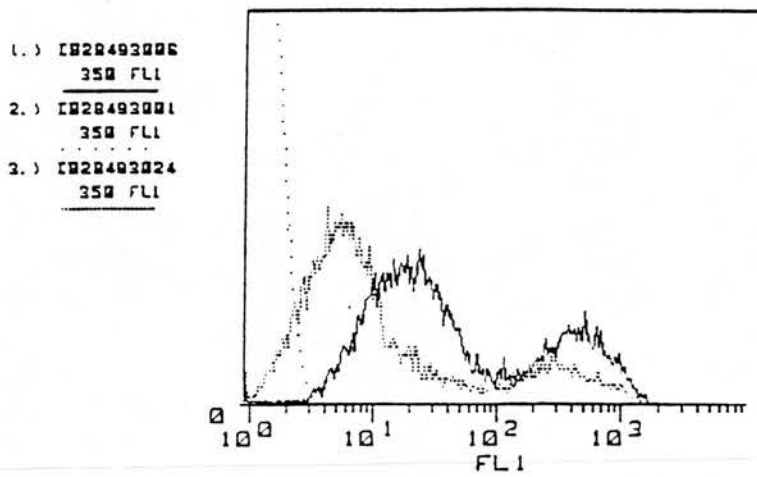


Table 3.2. Comparison of osmotic shock lysis and tris-NH₄Cl hypotonic lysis in peripheral blood leukocytes from control animals (n=7). Results are expressed as median and ranges of percentages of positively stained cells with the corresponding MAbs by reference to a negative control (unstained cells).

MAb	OSMOTIC SHOCK	TRIS NH₄Cl
CD4	22.3 (15.9-42.5)	33.4 (26.7-46.3)
CD8	4.1 (0.8-16.6)	3.1 (1.1-13.7)
γδ	5.2 (2.9-18.5)	5.1 (2.8-15.1)
CD5	22.0 (12.8-43.2)	33.4 (27.2-45.3)
VPM8	64.2 (32.3-88.7)	46.2 (27.0-75.4)

Not significant $p > 0.05$

3.4.3 BALF CYTOLOGY

According to the histologic criteria the following cell types were identified on cytocentrifuge preparations stained with Leishman's stain: alveolar macrophages, lymphocytes, neutrophils, eosinophils, mast cells, epithelial cells and erythrocytes.

Alveolar macrophages (Figs. 3.4 and 3.5). The population of alveolar macrophages was quite heterogeneous, including small, monocyte-like cells with a round to oval shape and few vacuoles, and larger cells containing two or more nuclei and numerous vacuoles.

Lymphocytes (Fig. 3.4) Small and large lymphocytes could be recognised. Small lymphocytes were round cells with concentric circular nuclei. Their cytoplasm was

scant, transparent or bluish in colouration and localised as a narrow strip around the nucleus. Large lymphocytes had more abundant and intensely blue staining cytoplasm. The variably shaped nucleus was usually eccentric and contained paler chromatin than the small lymphocytes. Occasional small basophilic granules were observed in the cytoplasm of both small and large lymphocytes.

Neutrophils (Fig. 3.4) were easy to recognise due to the typical lobulations of their nuclei (from two to five depending on their maturity state). Their cytoplasm was eosinophilic and usually contained numerous granules.

Eosinophils were characteristic cells with a bilobed nucleus (although occasionally monolobed). Uniformly sized eosinophilic granules were present within the cytoplasm, often obscuring the nuclear morphology. Disrupted eosinophils were occasionally recognised as a free nucleus surrounded by the eosinophilic granules.

Mast cells (Fig. 3.5) were readily recognised due to their oval, centrally located nucleus and numerous basophilic cytoplasmic granules, although they were heterogeneous with respect to size and granularity. Occasionally, to confirm the identity of these cells, a specific staining technique 'toluidine blue' (Appendix III.4) was necessary as their granules stained metachromatically with this dye.

Epithelial cells recovered in the BALF seemed to be closely correlated to the degree of trauma caused by the endoscope during BALF collection. Samples from animals which coughed during BALF collection contained higher numbers of epithelial cells. The first BALF aspirate contained more epithelial cells than subsequent aspirates. Two types of epithelial cells were distinguished, namely ciliated columnar epithelial cells and cuboidal epithelial cells. The former had a prominent brush border and a basal nucleus. These cells were recognised individually, although pairs and small clusters were more common. The latter were variable in size with a round centrally

Figure 3.4. Photomicrograph of BALF cells from a control sheep showing five alveolar macrophages (M), a neutrophil (N) and a small lymphocyte (L), x 1000.

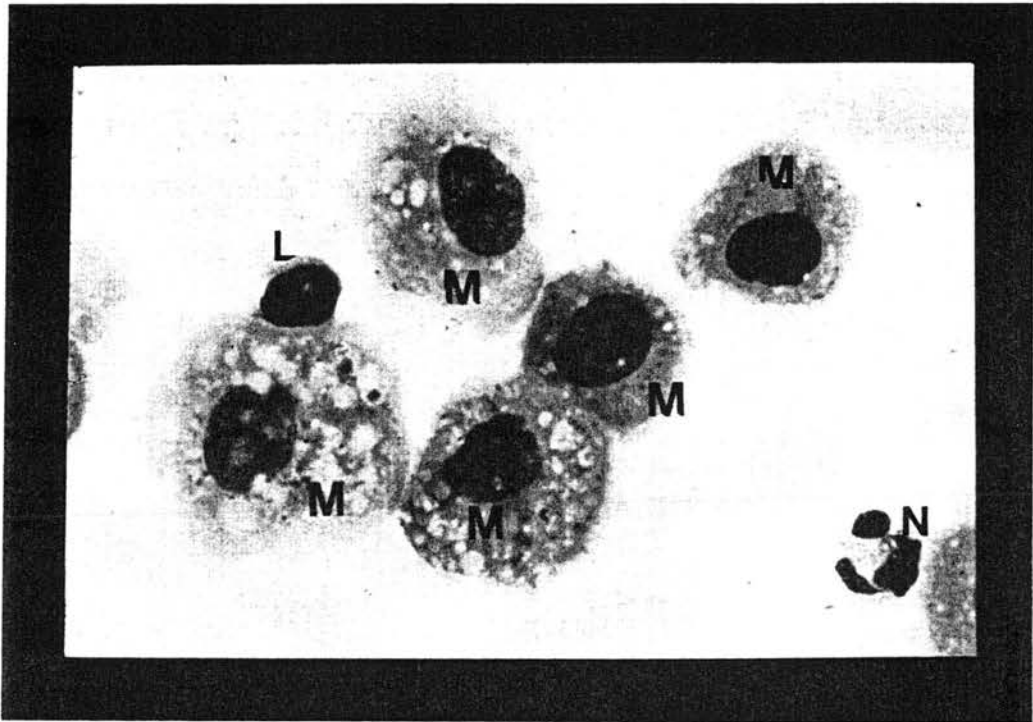
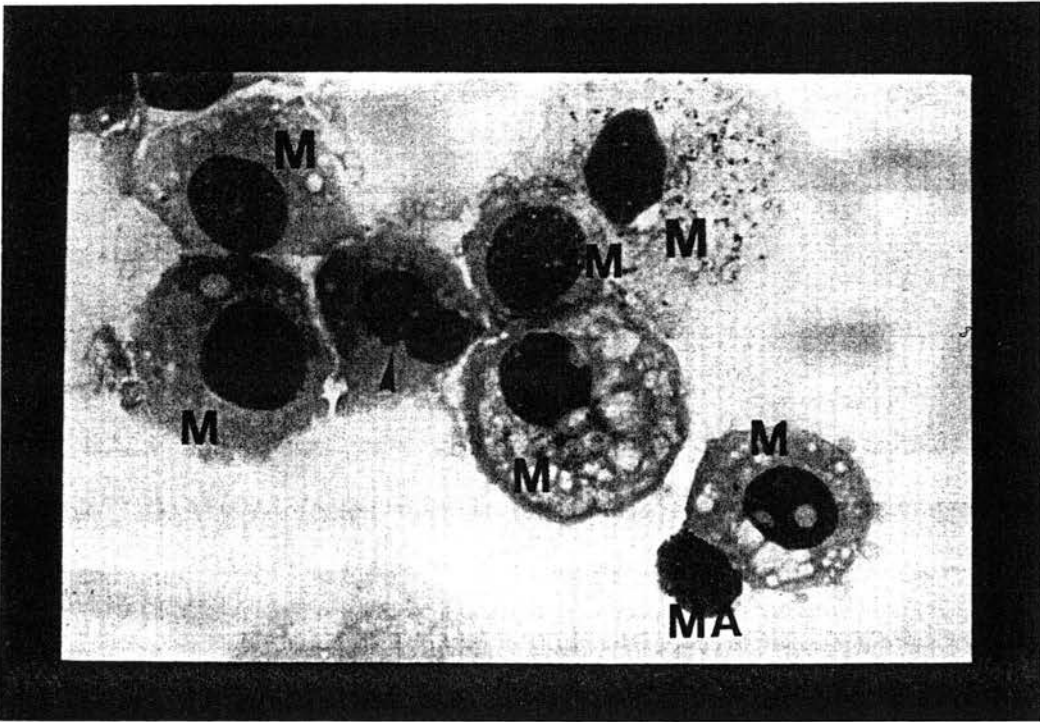


Figure 3.5. Photomicrograph of BALF cells from a control sheep showing seven alveolar macrophages (M), one of them binucleated, and a mast cell (MA), x 1000.



located hyperchromic nucleus and a shape varying from round to square. These cells, when single, were considered difficult to differentiate from alveolar macrophages.

3.4.4 BALF RECOVERY VOLUME

There were no significant differences in the volumes of BALF recovered from either group of animals (Table 3.3).

Table 3.3. Percent recovery of BALF from control and MVV-infected animals.

	CONTROL <u>(n=13)</u>	MAEDI <u>(n=12)</u>
	62.5	53.3
	65.0	71.5
	67.5	56.5
	70.0	46.5
	66.6	60.0
	68.0	53.3
	73.5	68.0
	51.0	72.2
	75.0	75.0
	45.5	55.0
	48.5	71.3
	68.5	66.6
	64.0	
MEDIAN	66.6	63.3
RANGE	(45.5-75.0)	(46.5-75.0)

Not significant ($p > 0.05$)

3.4.5 TOTAL AND DIFFERENTIAL BALF CELL COUNTS

Total BALF cell counts were similar in both MVV-infected animals and controls (Table 3.4). MVV-infected animals had significantly higher percentages of lymphocytes and neutrophils ($p < 0.05$) (Figs. 3.6 and 3.7) and significantly decreased percentages of alveolar macrophages and eosinophils ($p < 0.05$) when compared with

Figure 3.6. Photomicrograph of BALF cells from a MVV-infected animal. Accumulation of lymphocytes (L), x 1000.

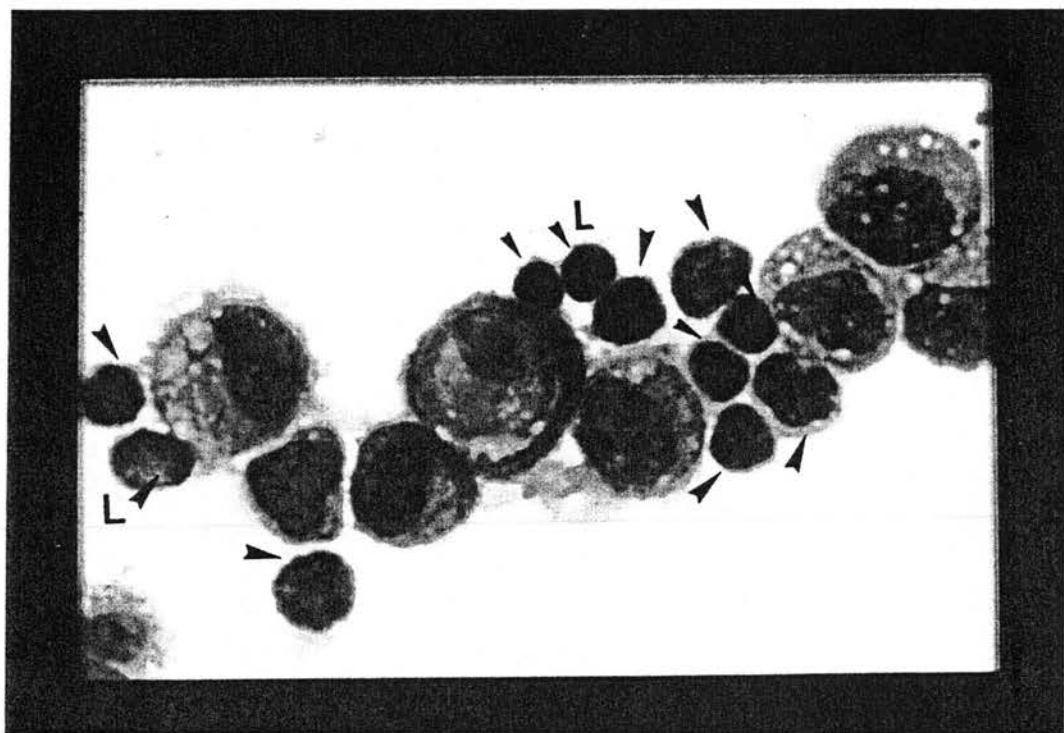
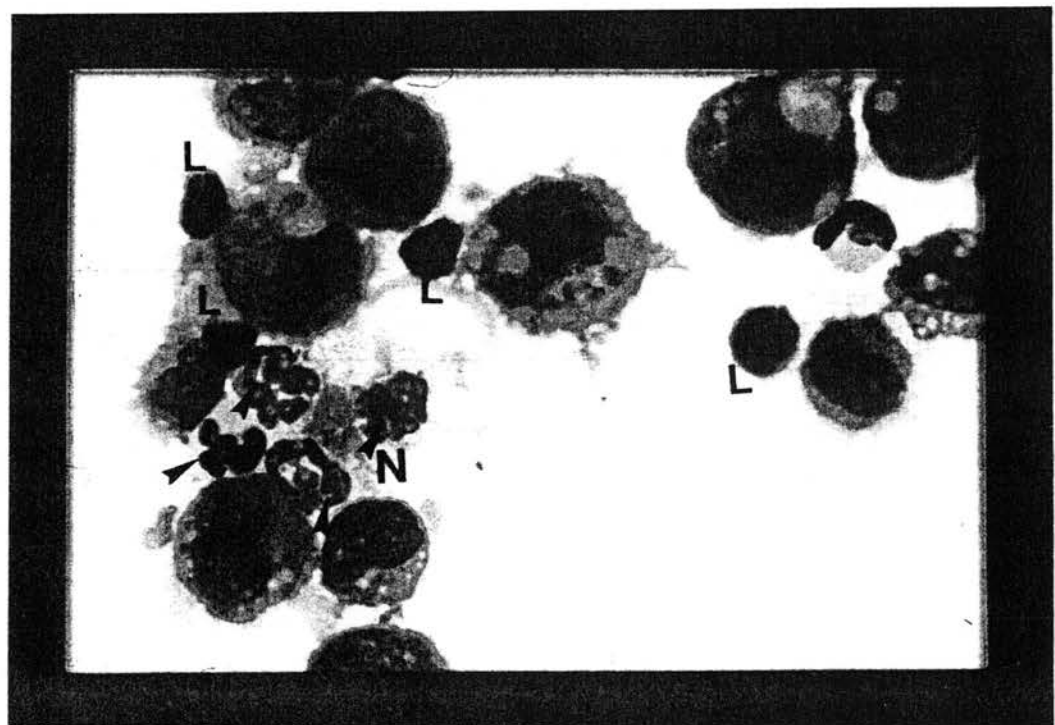


Figure 3.7. Photomicrograph of BALF cells from a MVV-infected animal. Accumulation of neutrophils (N) and lymphocytes (L), x 1000.



controls (Table 3.5). MVV-infected animals had significantly higher absolute numbers of lymphocytes ($p < 0.001$), neutrophils ($p < 0.001$) and alveolar macrophages ($p < 0.05$) (Table 3.6).

Table 3.4. Total BALF cell counts (cells/ml) for controls and MVV-infected animals (median and ranges).

CONTROL (n=13)	1.00×10^7	$(0.64- 2.76) \times 10^7$
MVV (n=12)	1.68×10^7	$(0.08- 3.15) \times 10^7$

Not significant ($p > 0.05$)

Table 3.5. BALF differential cell counts for control and MVV-infected animals (median percentages and ranges).

	<u>CONTROL (n=13)</u>	<u>MVV (n=12)</u>
NEUTROPHILS	1 (0-3)	5 (1-3)*
LYMPHOCYTES	5 (3-20)	13 (6-57)*
MACROPHAGES	86 (79-91)	69 (37-91)*
EOSINOPHILS	4 (0-11)	1 (0-9)*
MAST CELLS	1 (0-2)	0 (0-2)

* Significantly different at $p < 0.05$.

Table 3.6. Absolute cell counts ($\times 10^5/\text{ml}$) in BALF from control and MVV-infected animals (median and ranges).

	<u>CONTROL (n=13)</u>	<u>MVV (n=12)</u>
NEUTROPHILS	1.0 (0.0-3.0)	9.2 (1.6-15.0)*
LYMPHOCYTES	5.0 (3.0-20.0)	21.8 (10.0-95.7)*
MACROPHAGES	86.0 (79.0-91.0)	116.7 (62.1-152.8) ⁺
EOSINOPHILS	4.0 (0.0-11.0)	1.7 (0.0-15.1)
MAST CELLS	1.0 (0.0-2.0)	0.8 (0.0-3.3)

⁺ Significantly different at $p < 0.05$

* Significantly different at $p < 0.001$

3.4.6 DIFFERENTIAL PB CELL COUNTS

No significant differences were observed between both groups of animals in the percentages of PB neutrophils, lymphocytes, monocytes or basophils (Table 3.7). Eosinophils were significantly increased in the control animals ($p < 0.05$).

Table 3.7. Differential cell counts in PB of control and MVV-infected animals (median percentages and ranges).

	<u>CONTROL (n=13)</u>	<u>MVV (n=12)</u>
NEUTROPHILS	37 (6-50)	41 (21-78)
LYMPHOCYTES	58 (38-84)	55 (21-76)
MONOCYTES	4 (1-9)	3 (0-9)
EOSINOPHILS	4 (1-9)*	0 (0-2)
BASOPHILS	0 (0-4)	0 (0-1)

* Significantly different at $p < 0.05$

3.4.7 BALF AND PB LYMPHOCYTE PHENOTYPE DISTRIBUTIONS

In BALF of MVV-infected animals, the most noticeable changes were a significant decrease ($p < 0.05$) in CD4⁺ T lymphocytes and a significant increase ($p < 0.05$) in CD8⁺ T lymphocytes. This resulted in a significant inversion ($p < 0.001$) of the CD4⁺/CD8⁺ ratio in BALF of MVV-infected animals. A significant decrease ($p < 0.05$) of CD5⁺ lymphocytes was also observed. The percentage of $\gamma\delta$ T cells in BALF did not show significant variations. The percentage of BALF B cells was increased in MVV-infected animals, albeit non significantly (Table 3.8, Fig 3.8).

No significant differences were found in PB lymphocyte subsets between control and MVV-infected sheep (Table 3.9). The CD4⁺/CD8⁺ ratio in PB was similar in both groups.

The lymphocytic phenotypes from BALF were compared with those from PB, with values for control and MVV-infected animals being analysed separately (Fig. 3.9). Significantly higher percentages of CD4⁺ and CD5⁺ cells ($p < 0.05$) were found in BALF of control and MVV-infected animals when compared with PB. PB contained higher percentages of B cells (marked with MAb VPM8) than BALF, although this was statistically significant only for the control group ($p < 0.05$).

There were no significant differences in the FSC or in the SSC values for BALF lymphocytes (Appendix I.3.1).

3.4.8 BALF MACROPHAGE PHENOTYPING

Most BALF macrophages expressed LCA, but none expressed CD4, CD8, $\gamma\delta$ -TCR, CD5 or IgG-light chain molecules. When analysing the quantitative fluorescence of unlabelled BALF macrophages, their frequency histogram appeared shifted to the right of unlabelled BALF lymphocytes. This was due to the inherent

Table 3.8. Lymphocyte subsets in BALF from control and MVV infected animals.

	CD45	CD4	CD8	$\gamma\delta$ TCR	CD5	B cells	MHC Class II	CD4/CD8 ratio
CONTROL (n=13)	86.9 (68.4 - 96.7)	54.6 (42.3 - 67.1)	18.8 (8.5 - 30.4)	8.3 (3.8 - 19.6)	53.9 (40.6 - 66.9)	21.1 (13.1 - 51.1)	62.4 (14.1-91.1)	3.0 (1.8 - 5.3)
MAEDI (n=12)	89.6 (73.3 - 98.9)	33.4* (10.6 - 67.1)	29.0* (2.9 - 68.3)	5.4 (1.8 - 25.8)	32.6* (10.7 - 57.6)	33.1 (11.9 - 92.6)	41.4 (12.2-69.6)	0.9# (0.3 - 4.0)

Results are expressed as median of percentages of positively labelled cells in lymphocyte gate with ranges in brackets

* Significantly different from controls at p<0.05 (Mann-Whitney non parametric rank test)

Significantly different from controls at p<0.001 (Mann-Whitney non parametric rank test)

Table 3.9. Lymphocyte subsets in peripheral blood (PB) from control and MVV infected animals.

	CD45	CD4	CD8	$\gamma\delta$ TCR	CD5	B cells	CD4/CD8 ratio	MHC Class II
CONTROL (n=13)	96.2 (92.2-98.7)	19.3 (9.4-45.5)	24.7 (7.5-41.6)	6.3 (3.2-9.9)	18.3 (9.6-31.1)	61.5 (17.5-65.8)	0.9 (0.2-3.7)	63.7 (51.4-87.7)
MAEDI (n=12)	98.1 (91.1-99.1)	19.8 (9.7-28.6)	29.1 (11.3-43.4)	6.5 (3.4-8.2)	20.1 (8.7-27.5)	46.7 (11.9 -65.3)	0.8 (0.3 - 2.1)	77.0 (48.3-90.2)

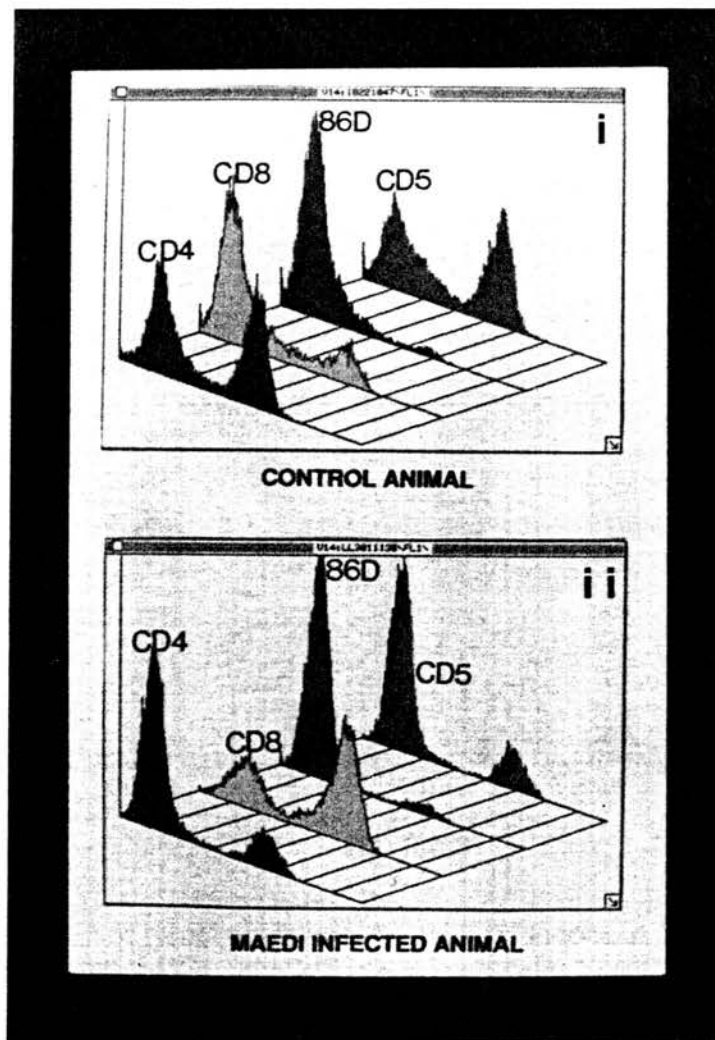
Results are expressed as median of percentages of positively labelled cells in lymphocyte gate with ranges in brackets

No significant differences from controls (Mann-Whitney non parametric rank test)

Figure 3.8. Summary of the lymphocyte phenotypic changes observed in BALF during MVV infection, with respect to a control animal. MVV-infected animals show a decrease in the proportion of CD4⁺ and CD5⁺ T lymphocytes, an increase in the proportion of CD8⁺ T lymphocytes, and no changes in the proportion of $\gamma\delta$ T lymphocytes.

(i) Lymphocyte subsets in BALF from a control animal.

(ii) Lymphocyte subsets in BALF from a MVV-infected animal.



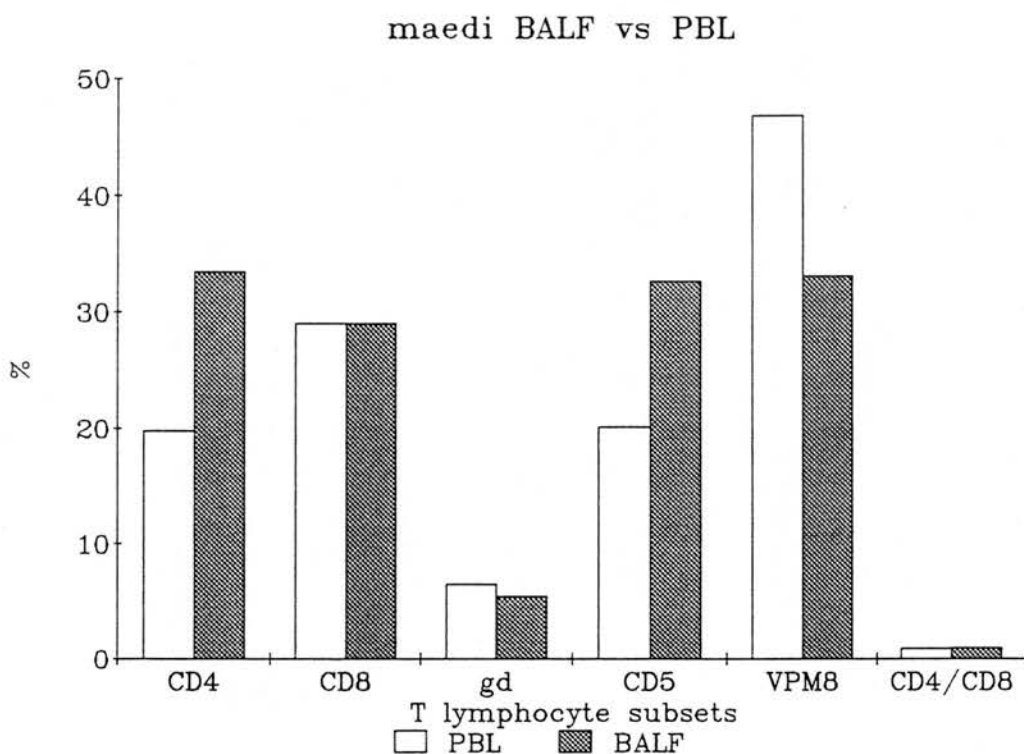
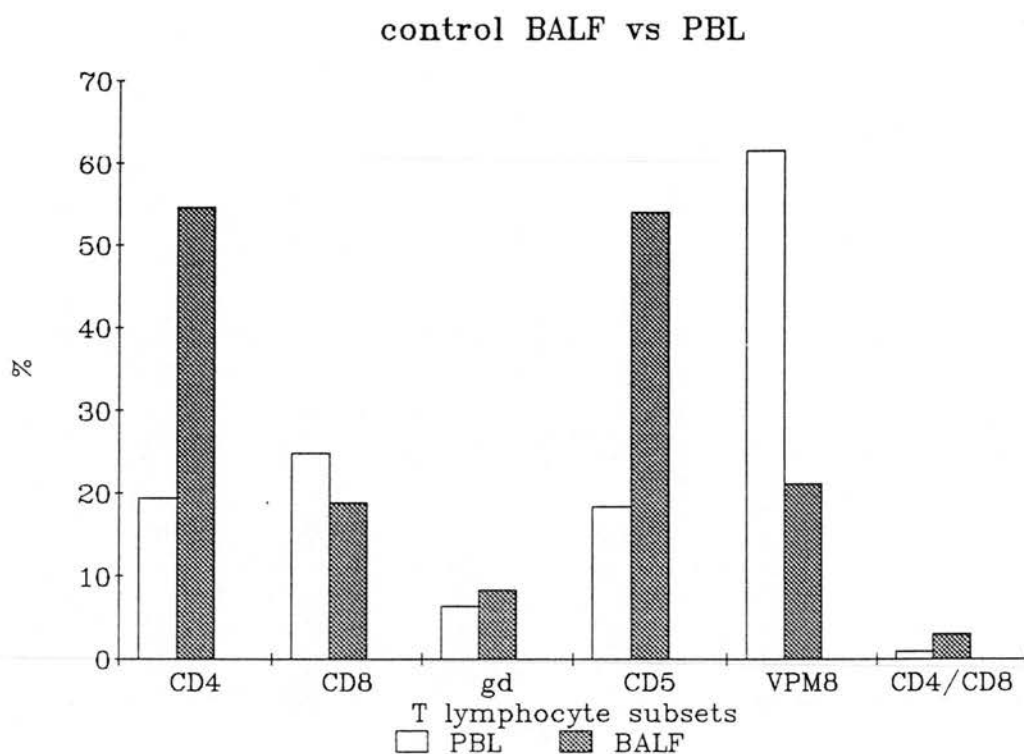


Figure 3.9. Comparison of lymphocyte phenotype distributions in BALF and PB from control (n=13) and from MVV infected (n=12) animals. Results are expressed as median of percentages of positively labelled cells. For results of statistical comparisons see section 3.4.7.

fluorescence of BALF macrophages, also referred to as 'autofluorescence'. This effect was readily overcome by decreasing the excitation wavelength of the flow cytometer prior to cytometric analysis (Parks *et al*, 1986).

The median FSC and SSC values for alveolar macrophages did not differ significantly between controls and MVV-infected sheep (Appendix I.3.2).

3.4.9 MHC CLASS II EXPRESSION BY BALF MACROPHAGES AND LYMPHOCYTES

The results of the expression of the MHC Class II molecules on BALF macrophages are shown in Table 3.10 and in Fig. 3.10. There was no significant difference in the percentages of positively staining macrophages between the two groups of animals (Appendix I.3.3), consistent with the finding that these antigens are constitutively expressed on most of the ovine alveolar macrophages (Nash *et al*, 1992). However, BALF macrophages from MVV-infected animals showed a significantly greater increase in MHC Class II molecule expression, as determined by the higher 'mode of fluorescence intensity' or 'mean channel number', following staining with the three reagents (Table 3.10, Fig. 3.10 and Appendix I.3.4).

Although BALF lymphocytes from both groups of animals expressed MHC Class II molecules, there were no significant differences in their expression in the two groups (Appendix I.3.5).

3.4.10 LFA-1 AND LFA-3 EXPRESSION BY BALF LYMPHOCYTES AND MACROPHAGES

As with the MHC Class II molecules, the 'mean channel number' was also used for the study of LFA-1 and LFA-3 on both BALF lymphocytes and macrophages. BALF lymphocytes from MVV-infected animals had significantly ($p < 0.05$) increased LFA-1 and LFA-3 (Table 3.11). BALF macrophages from MVV-infected animals showed increased expression of both molecules and this was significant for LFA-3 ($p < 0.05$) (Table 3.12, Fig. 3.11).

Table 3.10. Intensity of MHC Class II expression on BALF macrophages from control and MVV infected animals.

	Pan Class II	VPM 37 (DR)	VPM 41 (DQ)
CONTROL (n=13)	57.1 (10.5 - 233.7)	37.1 (5.87 - 51.26)	43.6 (9.4 - 195.1)
MAEDI (n=12)	251.2* (41.3 - 827.3)	98.2# (33.2 - 242.3)	175.0* (39.8 - 270.0)

Results are expressed as median of the value of mode channel number of fluorescence intensity with ranges in brackets.

* Significantly different from controls at $p < 0.05$ (Mann-Whitney non parametric rank test)

Significantly different from controls at $p < 0.001$ (Mann-Whitney non parametric rank test)

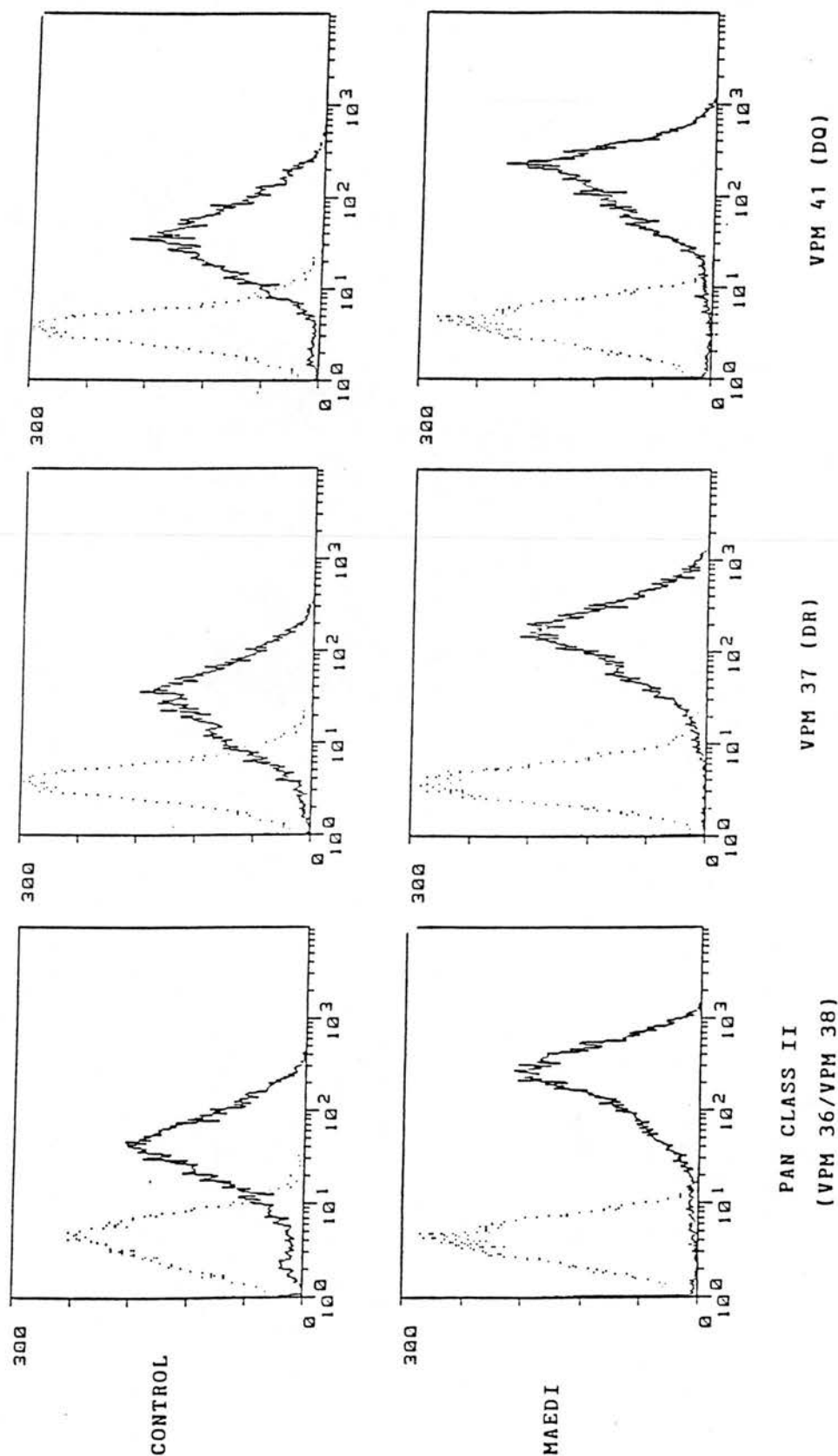


Figure 3.10. Fluorescence histograms of MHC Class II expression on BALF macrophages from a control and a MVV-infected sheep. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and linear cell frequency is given on the ordinate. (...) Negative control. Fluorescence profiles are shifted to the right in MVV-infected animals in both cases.

Table 3.11. LFA-1 and LFA-3 expression on BALF lymphocytes from control and MVV-infected animals. (Median mean channel number of fluorescence intensity and range).

	<u>LFA-1</u>	<u>LFA-3</u>
CONTROL	188.1 (47.7 - 312.0)	42.8 (12.1 - 81.9)
MAEDI	255.8* (76.3- 360.4)	70.9* (51.3 - 126.5)

* Significantly different from controls at $p < 0.05$.

Table 3.12. LFA-1 and LFA-3 expression on BALF macrophages from control and MVV-infected animals. (Median mean channel number of fluorescence intensity and range).

	<u>LFA-1</u>	<u>LFA-3</u>
CONTROL	29.8 (12.1 - 79.1)	31.0 (26.1 - 98.2)
MAEDI	52.21 (15.0- 117.7)	97.2* (37.0- 175.0)

* Significantly different from controls at $p < 0.05$.

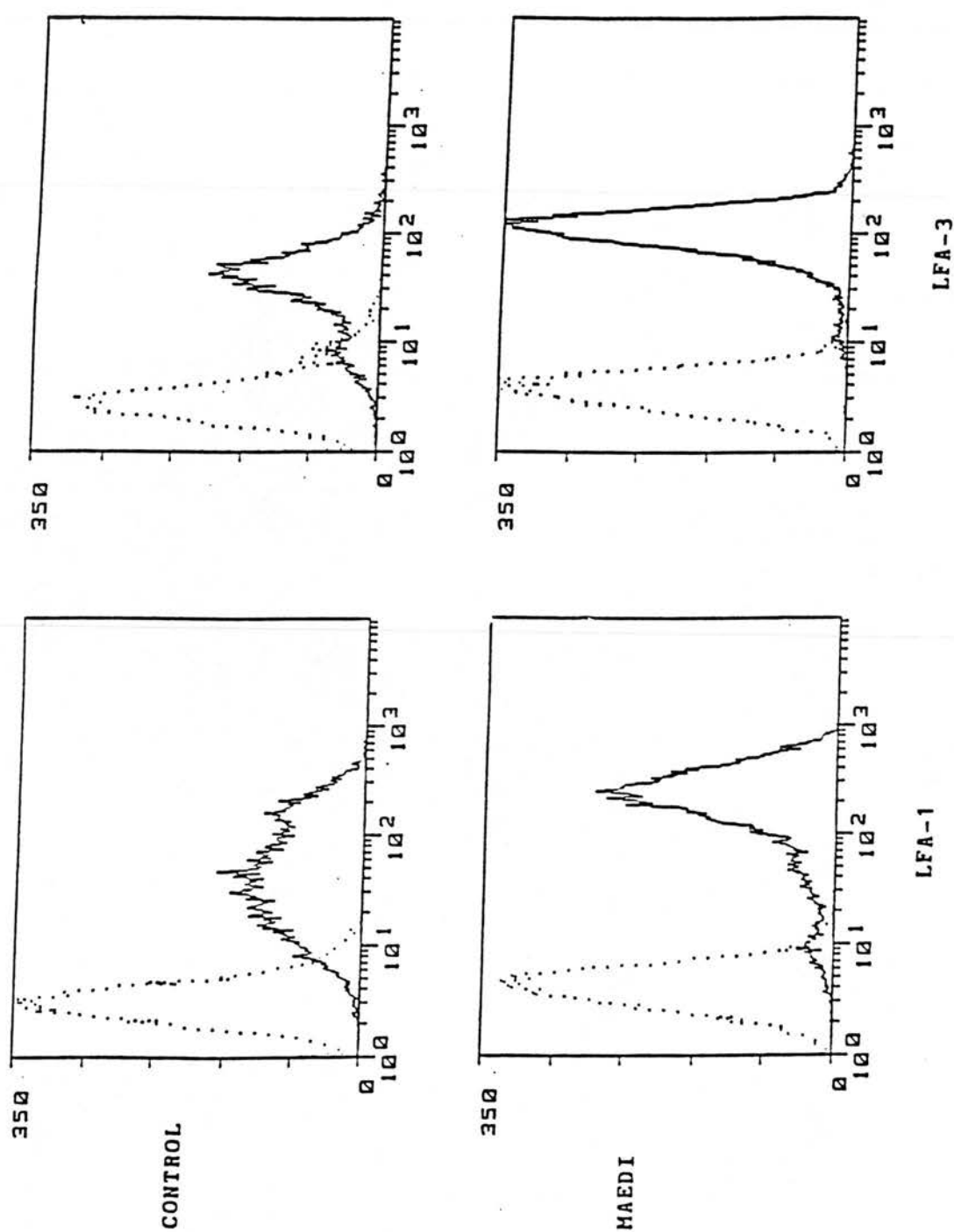


Figure 3.11. Fluorescence histograms of LFA-1 and LFA-3 expression on BALF macrophages from a control and a MVV-infected sheep. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and linear cell frequency is given on the ordinate. (...) Negative control. Fluorescence profiles are shifted to the right in MVV-infected animals in both cases.

3.5. DISCUSSION

The sampling of the sheep lung by BAL reflects accurately the immune response occurring in the affected organ. It also offers many advantages over previous analyses of the immune response in man and animals which used peripheral blood lymphocytes (PBL), spleen or lymph node cells (Meeusen *et al*, 1988; Prior and Haslam, 1992).

Characterisation of the BALF cellular immune response during the course of natural MVV infection has been previously described (Cordier *et al*, 1990; 1992).

The present study is an extension of the work of Cordier and others (1990). Results in the present work show that BALF cells from MVV-naturally infected sheep have different phenotype profiles and different levels of expression of some cell surface molecules from uninfected controls. This is concordant with the results of Cordier and others (1990). These alterations could play a key role in the pathogenesis of MVV in the lung and their investigation may provide a better understanding of the pulmonary pathogenic mechanisms of lentiviruses.

The combination of the BAL technique *in vivo* with single colour immunofluorescent labelling with MAbs and flow cytometric analyses has enabled this work to be performed without sacrifice of the animals. Future studies could be performed using the same animals to further investigate this disease.

The total BALF cell counts of MVV-infected animals were not significantly different from those of controls. This is in contrast to the increased BALF cellularity noted in the study of Cordier and others (1990). This discrepancy could be a consequence of differences in the two studies. Firstly, the present study used MVV seropositive sheep, while Cordier and others used lungs collected from an abattoir which had histological evidence of MVV infection, despite the histopathological

lesions of maedi not being pathognomonic (Cutlip *et al*, 1978; De Boer *et al*, 1978). Secondly, it is possible that the sheep used in both studies were in different stages of disease. Thirdly, as the age of the sheep used by Cordier and others was not stated, an age difference in the populations studied could account for differences in the results of the two studies.

Differential BALF cell counts (Table 3.5) of control animals were similar to those previously reported for normal ovine lungs (Burrells, 1985b). MVV-infected animals had significantly increased percentages of BALF lymphocytes and neutrophils and reduced percentages of BALF macrophages. While Cordier and others (1990), also described increased percentages of BALF neutrophils in MVV-infected sheep, increased percentages of BALF lymphocytes and reduced percentages of BALF macrophages were not observed.

The finding of significantly increased percentages of BALF lymphocytes and neutrophils is concordant with interstitial lung diseases in man and animals and in pulmonary AIDS (Weinberger *et al*, 1978; Martin *et al*, 1983).

Cordier and others (1990) found increased secretion of neutrophil chemotactic activity (NCA) by BALF cells from MVV affected animals. This cytokine could account for the amplified recruitment of neutrophils in the affected lungs. The pathogenic role of these neutrophils in the lung, if any, remains to be determined. While in sheep (Weiss *et al* 1983), monkeys (Kazmierowski *et al* 1977), dogs (Cohen and Batra 1980; Damiano *et al* 1980), horses (McGorum *et al*, 1993) and man (Von Essen *et al* 1991), repeated BAL may elicit a transient migration of neutrophils into the lungs, this phenomenon cannot explain the increased number of neutrophils in BALF from MVV-infected animals as only a single lavage was performed in this study.

Increased numbers of neutrophils in the alveoli is also a common finding in pulmonary bacterial infection (Damiano *et al*, 1980). The BALF neutrophilia noted in the present study was unlikely to have resulted from such a bacterial infection, as clinical and haematological examinations of these animals did not indicate the presence of any other significant concomitant pulmonary infection unrelated to MVV infection. Routine bacteriological culture of BALF was not performed because the frequent contamination of BALF by oropharyngeal organisms renders this technique of limited value in diagnosing pulmonary bacterial infections (Bartlett *et al*, 1976).

BALF from MVV-infected sheep had a significant lymphocytosis (Table 3.5), consistent with the finding that lymphocytes are the main mononuclear cell type present in MVV pulmonary lesions (Georgsson and Palsson, 1971). The increased BALF lymphocyte population could have resulted from recruitment of lymphocytes from PB and from other lymphoid organs, or from a local proliferation of lymphocyte populations within the lung. IL-2 could be involved in a proliferation of preexisting T cells (Hunninghake *et al*, 1983; Pinkston *et al*, 1983).

The numbers of lymphocytes in BALF from normal sheep and the comparison of lymphocyte phenotype distributions within BALF and PB has been recently established (Burrells and Sutherland, 1994). T lymphocytes account for almost the whole lymphocyte population in the lungs of healthy sheep, while B lymphocytes generally represent less than 10%. The present findings were concordant with these previous investigations.

Decreased percentages of BALF macrophages were the result of proportional increases in other cell populations in BALF. When reported as absolute concentrations rather than percentages, the numbers of BALF macrophages were significantly higher

than those of controls. A macrophage alveolitis has been described in some patients with AIDS (Agostini *et al*, 1991).

The increased percentages of neutrophils and lymphocytes and reduced percentages of macrophages seen in BALF of the MVV-infected animals appeared to be a local phenomenon within the lung, as these animals had no significant alterations in the numbers of circulating neutrophils, monocytes or lymphocytes.

There were no significant differences in the proportion of BALF mast cells between both groups of animals. The specific role of these cells in the ovine lung has not yet been determined.

The percentages of BALF eosinophils in the control and MVV-infected animals were within the normal range previously reported (Begin *et al*, 1981; Rola-Pleszczynski *et al*, 1981; Burrells and Williams, 1986), however, MVV-infected animals had an unexplained reduction in the proportion of eosinophils when compared with controls.

Natural infection with MVV was accompanied by a significant decrease in the BALF lymphocyte CD4⁺/CD8⁺ ratio, this decrease being due to both a significant increase in CD8⁺ T lymphocytes and a significant reduction in CD4⁺ T lymphocytes. Cordier and others (1990) had previously reported a decreased BALF lymphocyte CD4⁺/CD8⁺ ratio in MVV infection. However, while they observed an increase of the CD8⁺ population, no significant change of the CD4⁺ population was found. Cordier and others used the anti-CD4 MAb, SBU-T4, while in the present study, a different anti-CD4 MAb, 17D.13 was employed. Anti-CD8 MAb SBU-T8 was common for both studies. CD4⁺/CD8⁺ ratio inversion has also been found in synovial fluid of sheep with MVV-experimentally induced arthritis (Harkiss *et al*, 1991). The decreased

BALF lymphocyte CD4⁺/CD8⁺ ratio in MVV-seropositive animals could indicate either a state of increased immunoreactivity or a phenomenon related to a state of immunotolerance. Changes in this ratio can also indicate imbalances in immune-reactivity (Tizard, 1986) or pathology associated with a variety of virus infections (Novotney *et al*, 1990).

Human AIDS related lymphoid interstitial pneumonia also show an analogous reduced CD4⁺/CD8⁺ ratio and a preponderance of CD8⁺ T cells (Venet *et al*, 1984; Kennedy-Stoskopf, 1989). Disease complications associated with AIDS are usually attributed to a total depletion of CD4⁺ T cells, predisposing the patient to opportunistic infections (Fauci *et al*, 1984). The depletion of CD4⁺ T cells in AIDS is present both in PB and in BALF (Agostini *et al*, 1993). In contrast, MVV-infected sheep had normal CD4⁺ T cells in PB, and significantly reduced, but not totally depleted CD4⁺ T cells in BALF. Recent studies have, however, suggested that immunocompromised lymphocytic function during MVV infection could predispose animals to opportunistic infections (Brodie *et al*, 1992). The different CD4⁺ T cell patterns characterising MVV and AIDS suggests that although both causal lentiviruses induce a lymphoid interstitial pneumonia, the pathogenesis of the two conditions show important differences.

The cause of the decreased percentage of CD4⁺ T lymphocytes during the course of MVV-infection remains unexplained. While HIV selectively destroys human CD4⁺ T cells (Rankin *et al*, 1988), MVV is not lymphocytotropic, therefore viral infection of ovine CD4⁺ does not occur (Gorrell *et al*, 1992). In MVV, CD4⁺ T lymphocytes could be destroyed following an interaction with virus antigen presenting cells, such as macrophages and dendritic cells via MHC Class II antigens. An inhibition of proliferation of CD4⁺ T cells, due to cytokines produced during the

interaction of infected macrophages and lymphocytes, could also account for the reduced numbers of CD4⁺ T lymphocytes (Narayan *et al*, 1988a).

MVV-infected sheep had increased BALF CD8⁺ ratios, as occurs in human AIDS (Venet *et al*, 1985; Semenzato, 1991) and in some types of hypersensitivity pneumonitis (Berman *et al*, 1990). The effect of the increased numbers of CD8⁺ T lymphocytes in MVV-infection is still unclear, although it has been suggested that the CD8⁺ T lymphocytic alveolitis in man could induce damage of the lung epithelium and cause clinical respiratory distress (Meignan *et al*, 1990). CD8⁺ T lymphocytes could induce lung damage by activating macrophages through the production of mediators such as γ -interferon (γ -IFN) (Kaufmann, 1988). This γ -IFN could cause expression of MHC Class II antigen in infected and uninfected macrophages potentiating the lymphoproliferative inflammatory disease in the lung (Kennedy-Stoskopf *et al*, 1985b). Furthermore, cytotoxic T cells could act bimodally either clearing virus or amplifying viral infections (Giorgi *et al*, 1987a; 1987b). In AIDS this dual function has also been proposed and therefore, this lymphocyte subset could either contribute to the tissue damage (Klatzmann and Gluckman, 1986) or serve as an inhibitory mechanism of HIV replication by production of a soluble factor (Walker *et al*, 1986).

The proportion of CD5⁺ cells in BALF from MVV-infected animals was also significantly decreased. As an almost total loss of cell membrane CD5 follows ovine lymphocyte activation (Hopkins and Dutia, 1990), this finding suggests that BALF T lymphocytes were activated during MVV-infection. The presence of activated lymphocytes in the BALF of infected animals indicates the presence of an ongoing local immune process and suggests that these lymphocytes could contribute to the genesis of lung tissue lesions through the interaction with other effector cells and the production of mediators (Mornex *et al*, 1984; Klatzmann and Gluckman, 1986).

Activated T lymphocytes have a well established role in AIDS by promoting the progression of HIV infection (Mahalingam *et al*, 1993), and in human pulmonary sarcoidosis through the release of certain lymphokines such as monocyte chemotactic factor and migration inhibitory factor (Mornex *et al*, 1985).

No significant differences were found between the BALF $\gamma\delta$ T lymphocytes of MVV-infected and control animals. Cordier and others (1992), obtained similar findings using a different MAb, namely SBU-T19, which has the same specificity as that used in the present study. While the biological function of $\gamma\delta$ T cells is not yet fully understood, protection of epithelial surfaces, participation in cytotoxicity and participation in macrophage proliferation and granuloma formation have been suggested (Augustin *et al*, 1989; Mackay and Hein, 1989; Modlin *et al*, 1989; Haas *et al*, 1990; Spits, 1991). The recent finding of increased $\gamma\delta$ -TCR cells in some patients with hypersensitivity pneumonitis (Semenzato, 1991) and in patients with HIV-1 infection (Agostini and Semenzato, 1990; Agostini *et al*, 1994) suggests they have a role in the pulmonary immune response. Human BALF $\gamma\delta$ T cells show different phenotypic characteristics when compared with those of blood, as they coexpress CD8 and the NK-related CD56 marker (Agostini and Semenzato, 1990).

LFA-1 and LFA-3 molecules were upregulated on BALF lymphocytes from MVV-infected animals. Both molecules are cell adhesion molecules with an active role in leucocyte circulation, activation and proliferation (Arnaout, 1990; Pardi *et al*, 1992). Thus, the increased expression of both LFA-1 and LFA-3 in MVV-infection could account for the pulmonary recruitment of circulating lymphocytes and their *in situ* activation, and also for the interstitial lymphoid proliferation. Furthermore, as LFA-1 expression by lymphocytes is increased on activation (Kurzinger *et al*, 1981; Nishimura and Itoh, 1988), the increased expression of this molecule on BALF

lymphocytes from MVV-infected animals suggests that these cells are activated in maedi.

No significant differences were found between the BALF B lymphocytes of MVV-infected and control animals, suggesting that pulmonary B cells do not have an important role in the pathogenesis of maedi. There are no previous studies examining BALF B cell percentages in MVV-infection. In AIDS, BALF B cells are increased and also present functional abnormalities (Chess *et al*, 1984; Young *et al*, 1985; Martinez-Maza *et al*, 1987).

To examine whether differences seen in the expression of some lymphocyte surface antigens between control and MVV-infected sheep were due to differences in the cell size or complexity, the FSC and SSC scatter measurements on BALF lymphocytes of the controls were compared with those of MVV-infected sheep as previously reported for PBL of patients with sarcoidosis (Shakoor and Hambkin, 1992). The fact that no significant differences in the mean FSC or SSC values were detected between BALF lymphocytes of both groups of animals indicated that the differences seen in the expression of some lymphocyte antigens were not due to differences in the lymphocytes size or complexity.

Cell surface phenotypes of ovine BALF macrophages from control and MVV-infected animals were similar. These cells expressed LCA, but not CD4, CD8, CD5 and $\gamma\delta$ -TCR molecules, consistent with the findings of Nash and others (1992). In contrast, BALF macrophages from normal humans and rats (Crocker *et al*, 1987) and some large macrophage-like cells from ovine afferent lymph (Mackay *et al*, 1988b) express CD4. CD4 and CD8 molecules are also expressed on cultured ovine macrophages derived from blood monocytes from both MVV-infected and control animals (Wei Cheng Lee, personal communication). In man, CD4 molecules on BALF

macrophages act as receptors for the HIV virus resulting in a low grade persistent infection of these cells *in vitro* (Plata *et al*, 1990). As CD4 is absent from ovine BALF macrophages, these molecules cannot act as receptor molecules for MVV; MHC Class II antigens have been shown to act as a component of the cellular receptor for ovine MVV (Dalziel *et al*, 1991).

BALF macrophages showed autofluorescence, similar to that of human BALF macrophages (Edelson *et al*, 1984). While all the causes of autofluorescence are not yet clear, this seems to be a function of cell size, cell type, excitation wavelength and emission detection range and is usually attributable to normal cell constituents such as flavin nucleotides and pyridine which tend to predominate in larger cells (Benson *et al*, 1979; Parks *et al*, 1986). Accumulation of ageing pigments within cells such as lipofuscin or ceroid which alter the cellular metabolism (Shapiro, 1983) and changes in the fluorescence of pyridine nucleotides following alteration in the intracellular redox state due to oxidant stress (Edelson *et al*, 1985) are also potential causes for the autofluorescence. In the present study, the problem of autofluorescence was minimised simply by performing flow cytometry using a reduced excitation wavelength.

The significant increased expression of MHC Class II molecules in BALF macrophages of MVV-infected animals seems to be an indicator of a state of activation in this cell population. This finding is concordant with the work of Cordier and others (1990), who also found increased spontaneous release of fibronectin and of NCA (as previously reported) by BALF macrophages.

The interstitial lung disease observed in MVV infection is probably mediated by activated macrophages expressing MHC Class II molecules. These cells are not only the target cells for MVV but, by presenting MVV antigen in association with MHC Class II antigens to T_H cells, could cause proliferation of CD8⁺ lymphocytes,

resulting in inflammation and tissue damage. Furthermore, as MHC Class II antigens are a component of the cellular receptor for MVV (Dalziel *et al*, 1991), increased expression of these molecules could promote the spread of the virus to uninfected macrophages, allowing persistence of the virus within the host.

Finally, MHC Class II molecule expression on macrophages is regulated by γ -IFN (Zink *et al*, 1987; Nash *et al*, 1992) which is produced during the interaction between the lentivirus-infected macrophages and lymphocytes (Narayan *et al*, 1985). The γ -IFN produced *in vivo*, by increasing the expression of MHC Class II antigens on BALF macrophages might contribute to the host lymphoproliferative response to MVV (Kennedy-Stoskopf *et al*, 1985b).

The lack of significant differences in the mean FSC and SSC values between both groups of animals confirmed that the greater expression of MHC Class II molecules found on BALF macrophages from MVV-infected animals was not attributable to variations in cell size or complexity (Shakoor and Hamblin, 1992).

LFA-1 molecule expression by BALF macrophages was not significantly affected by MVV-infection. In AIDS, a decreased percentage of LFA-1⁺ BALF macrophages has been observed (Bene *et al*, 1988).

When compared with PB, BALF from control sheep had significantly higher percentages of CD4⁺, CD5⁺ and lower proportions of B cells, consistent with the findings of Burrells and Sutherland (1994). Similar differences in PB and BALF lymphocyte phenotype have been reported in man (Yamada *et al*, 1986) and horses (McGorum *et al*, 1993). Nelson and others (1990) reported differences between lymphocytic phenotypes in rat lung vascular beds and PB. Furthermore, functional differences between lung and blood lymphocytes have been demonstrated (Nelson *et al*, 1990). These findings indicate that lymphocyte phenotype compartmentalisation may occur in normal individuals.

Although correlated phenotypic changes in PB and synovial fluid lymphocytes in MVV-infected animals have been described (Kennedy-Stoskopf *et al*, 1989), in this study, alterations in the distribution of lymphocyte phenotypes in BALF from MVV-infected animals were distinct from and not associated with lymphocyte phenotype changes in PB. Furthermore, phenotypes of PBL from control and MVV-infected sheep showed considerable individual variation, suggesting that the determination of PBL phenotypes is not a valuable indicator of the pathological processes occurring in the lung during the course of MVV infection. These findings indicate that local pulmonary immune mechanisms may be operative without corresponding systemic involvement (Salvaggio and Karr, 1979; Westermann and Pabst, 1990). Consequently, phenotypic evaluation of BALF lymphocytes provides a more accurate assessment of the pathological processes occurring during the course of MVV infection than evaluation of PBL. Furthermore, many factors other than the presence of disease, including age, sex, stress and treatment with drugs can influence PB lymphocyte phenotype distributions (Westermann and Pabst, 1990). Despite these reservations, functional and phenotypic evaluations of human PB T-lymphocytes have proved useful in the assessment of immunological status in several diseases (Moretta *et al*, 1979; Brouet *et al*, 1980).

To summarise, the altered BALF T cell phenotypic profiles of MVV-naturally infected sheep, together with the upregulation of MHC Class II molecules on BALF macrophages and of LFA-1 and LFA-3 molecules on BALF T lymphocytes would suggest that activation of BALF lymphocytes and macrophages occurs during natural MVV-infection. Further studies into the state of activation of BALF T lymphocytes in MVV-infection were performed and are described in Chapter 4.

CHAPTER 4: FLOW CYTOMETRY STUDY ON THE ACTIVATION OF BALF AND PB T LYMPHOCYTES DURING THE NATURAL COURSE OF MVV INFECTION.

4.1. SUMMARY

A study on the stage of activation of lymphocytes recovered by BAL and from PB in MVV-infected (n=7) and control (n=7) sheep was performed using dual-colour flow cytometry. The lymphocyte population was defined by its light scattering properties. Three parameters of lymphocyte activation were assessed, namely cell size and complexity, cell surface interleukin-2 receptor (IL-2R) expression, and DR and DQ MHC Class II molecule expression. MVV-infected animals showed upregulation of DR and DQ MHC Class II molecules on BALF lymphocytes, without changes in cell size and complexity or increased expression of IL-2R. No evidence of PB lymphocyte activation was detected. These findings suggest that an impaired process of lymphocyte activation occurs during natural MVV-infection.

4.2. INTRODUCTION

Although MVV seems not to be lymphotropic (Gorrell, 1992), lymphocytes are the main cell type which accumulate in the lung during infection (Narayan and Clements, 1989). The putative role of T lymphocytes in human lung immune response and immunologic lung diseases has given rise to many functional and phenotypic studies of this particular cell type (Davidson *et al*, 1985). All the lymphocyte categories, such as T, B and NK cells are present in the lung, but research work has mainly concentrated on T lymphocytes as they form the predominant lymphocyte population in epithelial lining fluid (Saltini *et al*, 1984). Pulmonary lymphocytes differ from circulating lymphocytes in that, even when resting, some appear to be partially activated, (Semenzato, 1991). Hence, experiments performed on unimmunised canine

lungs have shown that while unstimulated lymphocytes comprise over 90% of the bronchoalveolar lymphoid cells, the remainder of the lymphoid cells are activated lymphocytes and very occasional plasma cells (Brownstein *et al*, 1980).

Previous studies of BALF cells from MVV-naturally infected sheep had shown altered T lymphocyte phenotypic profiles. All these changes, together with an upregulation of LFA-1 (Kurzinger *et al*, 1981) and LFA-3 (Mackay *et al*, 1988b) molecules on BALF T lymphocytes suggest an active T lymphocyte involvement during the respiratory form of the disease (Watt *et al*, 1992b; Lujan *et al*, 1993).

In HIV infection, T cell activation is a consistent and early feature (Mahalingam *et al*, 1993). It is known that the accumulation of lymphocytes in the lung not only plays a passive role in the alveolitis, but these lymphocytes by undergoing activation might contribute to the development of lesions in the pathogenesis of lymphocytic alveolitis through interactions with other effector cells (Mornex *et al*, 1985). Activation of lung T cells is also considered a major factor in the pathogenesis of human interstitial lung disease (ILD) (Crystal *et al*, 1981; Ginns *et al*, 1982; Keogh and Crystal, 1982; Mornex *et al*, 1984; 1985). Infection with MVV provides an animal model of diffuse fibrosing ILD in humans (Cordier *et al*, 1989). The aim of this study was to determine the degree of activation of PB and BALF T lymphocytes of MVV-infected sheep.

MAbs and phenotypic analysis of activation markers have been successfully used in recent years to probe for cell surface molecules involved in T lymphocyte activation (Yu *et al*, 1980; Hunig *et al*, 1986) and for the assessment of the immunological status of some diseases such as idiopathic myositis (Miller *et al*, 1990). In the latter study, cell size and the presence of MHC Class II and IL-2R antigens were the parameters of T cell activation. The presence of these markers has

been reported to be good evidence of lymphocyte activation in man, both *in vivo* (Yachie *et al*, 1983; Morris *et al*, 1993) and *in vitro* (Krown *et al*, 1983).

4.2.1 T LYMPHOCYTE ACTIVATION

Lymphocytes recognise and respond to foreign antigens by undergoing a series of multiple transformations, all these changes being grouped together under the name of 'activation'. The process of activation involves a series of cellular and lymphokine-mediated events, depends upon the participation of effector cells such as mononuclear phagocytes and other leukocytes and culminates in T cell proliferation and the emergence of specific effector T lymphocytes (Goodman, 1982; Samelson, 1989).

Most of the current knowledge of the cascade of molecular events in T cell activation is derived from *in vitro* experiments in which T cells can be stimulated in a controlled manner, and their responses measured accurately. These cellular events, although still incompletely understood, comprise the following interconnected steps;

1) *Early signal transduction events*, include membrane phospholipid breakdown, elevated protein kinase C activity and increased cytoplasmic calcium. After TCR antigen-binding, intracellular signalling is transduced to the cytoplasm through the CD3 complex of surface molecules (June, 1991). The TCR/CD3 complex functions, thus, as a signal transducing unit, being critical to T cell recognition by conferring antigen-specificity and initiating the cascade of biochemical events for T cell activation (Clevers *et al*, 1988). Apart from the TCR/CD3 complex, multiple additional accessory molecules, such as CD4, CD8, CD45, CD28 and CD2 can fully activate T cells in combination with a TCR/CD3 mediated signal (Geppert *et al*, 1990).

2) *Transcriptional activation of a variety of genes*; the transcription or expression of the IL-2R gene enables the proliferation of T cells, thus controlling both

the initiation and the maintenance of the immune response. After the transcription of the IL-2 and IL-2R genes, there is activation of the genes coding for some other lymphokines, such as γ -IFN, TNF- α and IL-4.

3) *Expression of new cell surface molecules* (i.e. MHC Class II molecules or transferrin receptor).

4) *Secretion of cytokines* (i.e. γ -IFN, TNF- α , IL-2, IL-3, IL-4, IL-5 and IL-6).

5) *Induction of mitotic activity*; functional and mitotic responses of T cells to antigenic stimulation last only for brief periods, and the responses decline as the antigen is eliminated.

4.2.2 PARAMETERS OF LYMPHOCYTE ACTIVATION

For the estimation of T lymphocytes activation, several individual parameters have been described (Mornex *et al*, 1985; Hancock *et al*, 1986);

1) Measurement of changes in RNA and DNA content of cells (as their increase characterises proliferating cells), either by flow cytometry or through [3 H] thymidine uptake.

2) Cell size, as large lymphocytes are regarded as an early indicator of activation.

3) Cytological features, such as size, have also been used to separate lymphoid cells into unstimulated lymphocytes, activated lymphocytes and plasma cells (Browsntein *et al*, 1980).

4) Presence of activation antigens, such as MHC Class II antigens.

5) Spontaneous release of lymphokines, i.e. IL-2.

6) Increased response to mitogens.

4.2.3 INTERLEUKIN-2 AND ITS RECEPTOR.

The production of IL-2 and expression of its receptor are main events in the T cell response (Reem and Yeh, 1984; Waldmann, 1986).

IL-2 or T cell growth factor, first described in 1976 as a factor promoting growth of T lymphocytes from normal human bone marrow (Morgan *et al*, 1976) is a glycoprotein with apparent MW 15,500 which is synthesised and secreted by T cells in the initial phase of their activation by antigens and mitogens or in mixed lymphocyte culture (MLC) in the presence of macrophage-derived interleukin-1 (IL-1) (Smith, 1980; Diamantstein *et al*, 1986; Kaczmarck, 1986). IL-2 binds to each cell with two distinct affinities, one high and one low. IL-2, through binding to high affinity IL-2R, exerts growth promoting and proliferation effects on activated T cells (Smith *et al*, 1979; Robb *et al*, 1981), proliferation and activation of NK cells (Henney *et al*, 1981), proliferation of lymphokine activated killer cells (Grimm *et al*, 1983), induction of cytotoxic T cell activity, promotion of B-cell proliferation and differentiation *in vitro* (Tsudo *et al*, 1984; Waldmann *et al*, 1984) and control of lymphokine production (Waldmann, 1986). The role of the low affinity receptor remains unknown (Dukovich *et al*, 1987).

4.2.4 THE USE OF IL-2R AS AN ACTIVATION MARKER

T lymphocyte proliferation is characterised by the appearance of new cell surface antigens termed 'T cell activation antigens'. These activation antigens are not present on resting T cells and each has a characteristic and reproducible time of appearance after T cell activation by mitogens. Based on their temporal appearance they have been classified as early, intermediate or late antigens (Cotner *et al*, 1983). According to this classification IL-2R is an 'early antigen' and its expression is considered to be a better and earlier indicator of lymphocyte activation than DNA synthesis, as IL-2R is required for DNA synthesis (Miyawaki *et al*, 1982; Cotner *et al*, 1983). Hence, the measurement of IL-2R reactive cells has been considered as a valid

parameter of early T cell activation (Prince and John, 1986; Famularo *et al*, 1990; Santos *et al*, 1991; Morris *et al*, 1993), appearing shortly after the *in vitro* activation of T cells (Larsson and Coutinho, 1979; Miller *et al*, 1990). The transition of T cells from a resting to an activated state is thus marked by the expression of IL-2R (Greene *et al*, 1984, 1986a; Kronke *et al*, 1984).

Although *in vitro* stimulated B cells and monocytes/macrophages and NK cells also express IL-2R (Waldmann, 1986) as they do it to a much lesser extent than activated T cells (Famularo *et al*, 1990) IL-2R can be regarded as T cell specific. IL-2R is a more sensitive activation marker than other T cell activation-associated antigens, such as the transferrin (Omary *et al*, 1980; Trowbridge and Omary, 1981) or the insulin receptor (Helderman and Strom, 1979), which are widely distributed in many proliferating cell types (Santos *et al*, 1991).

Most of the knowledge about the structure, function and expression of the human IL-2R was obtained through the production of the anti IL-2R, anti-Tac or anti-CD25 MAb by Uchiyama and others (1981). A MAb specific for bovine IL-2R α -chain, which crossreacts with ovine CD25, has also been recently produced (Naessens *et al*, 1992).

4.2.5 THE USE OF MHC CLASS II ANTIGENS AS ACTIVATION MARKERS

Human MHC Class II antigens, which are constitutively expressed on some cell types such as B cells, monocytes and dendritic cells (Kaufman *et al*, 1984; Kappes and Strominger, 1988), fall into the category of activation antigens on peripheral T cells (Mittler *et al*, 1983).

In man, the appearance of MHC Class II antigens on T cells is a relatively late event after mitogen-dependent activation of T cells (Ko *et al*, 1979), being expressed in higher density in the G₂ phase of activated T cells (Matsui *et al*, 1986). Therefore, these antigens are regarded as 'late antigens' (Cotner *et al*, 1983).

However, there is considerable inter-species variation in the expression of MHC Class II by T lymphocytes. Hence, while human T lymphocytes synthesise and express MHC Class II antigens only after activation (Ko *et al*, 1979; Robbins *et al*, 1988), feline (Rideout *et al*, 1992), canine (Deeg *et al*, 1982) and equine (Crepaldi *et al*, 1986; Monos *et al*, 1989) MHC Class II molecules are constitutively expressed by T-lymphocytes. In mice, there is some controversy regarding whether the MHC Class II antigens expressed on activated T cells are synthesised by T cells (Lorber *et al*, 1982) or adsorbed from the stimulating cell population (Singh *et al*, 1984). In sheep, expression of MHC Class II molecules is dependent both on the age of the animal and the physiological location of the T cells (Dutia *et al*, 1993). A proportion of ovine resting peripheral (Dutia *et al*, 1993; Burrells and Sutherland, 1994) and efferent lymph (Hopkins *et al*, 1993b) T cells express DR⁺ MHC Class II molecules. Expression of MHC Class II DQ⁺, however, can be considered as an activation marker in the sheep (Dutia *et al*, 1993; Hopkins *et al*, 1993b).

4.2.6 THE USE OF DUAL COLOUR FLOW CYTOMETRY TO STAGE BALF T LYMPHOCYTE ACTIVATION

The study of BALF T lymphocyte activation by flow cytometry, unlike estimation of mediator release, allows a direct quantification of the numbers of activated cells. The combined qualitative and quantitative determination of the diversity of activation markers expressed by BALF T lymphocytes might prove useful for the assessment of the possible relationship between these markers and disease progression. It could also provide criteria for staging the intensity of the lymphocytic alveolitis, giving a better method for studying the ongoing local immune response in the lung during the course of MVV infection.

When trying to identify the specific function of a cell, the presence, absence or degree of expression of multiple markers on the surface of that cell provides more

information about its specific function than the study of the presence of a single antigenic determinant (Carter and Meyer, 1990).

Two colour flow cytometry enables simultaneous measurement of the fluorescence intensity of two antigens on each cell, allowing thus a correlated, multiparametric evaluation of markers related to cell function.

The use of dual and multiple colour immunofluorescence, although available for some time, did not play an important role in clinical investigation until recently due to technical problems and the lack of suitable fluorochromes. Today, the availability of new dyes, such as fluorescein-phycoerythrin, has made dual colour immunofluorescence a valuable technique.

4.3. MATERIALS AND METHODS

4.3.1 ANIMALS

Seven adult Texel ewes (age range 3-7 years), seropositive to MVV by the AGIDT, and seven breed, sex and age matched seronegative controls ewes were used (Chapter 2). Different animals from those employed in Chapter 3 were selected for this study.

4.3.2 SAMPLE COLLECTION

BALF and PB sample collection and processing were performed as previously described in Chapter 2. A control and a MVV seropositive animal were sampled on each occasion.

4.3.3 MONOCLONAL ANTIBODIES AND REAGENTS

MAbs and the immunoconjugates used in this study are listed in Table 4.1. Biotinylated MAbs were conjugated to biotin according to the method of Hudson and Hay (1989).

R-phycoerythrin-streptavidin (PE-SA) complex (Amersham International, Amersham, U.K.) was used as a second step reagent to complex with the biotinylated MAbs. Rabbit anti-mouse IgG₁ FITC (The Binding Site, Birmingham, UK) was used to label unconjugated primary MAbs, since they were all of the IgG₁ isotype. Rabbit anti-rat IgG_{2b} FITC (Serotec, Kidlington, UK) was employed to label the unconjugated primary rat MAb SW73.2.

All the MAbs and immunoconjugates were used at previously determined optimal working dilutions (Table 4.2).

4.3.4 DUAL COLOUR LABELLING TECHNIQUE

The staining procedure employed has been previously described (Chapter 2).

4.3.5 DUAL COLOUR FLOW CYTOMETRIC ANALYSIS

Quantification of antigen expression was as described by Hopkins and others (1989), using a FACSCAN flow cytometer (Becton Dickinson Ltd, Los Angeles, California) equipped with Consort 30 version F software. A 488-nm light (200 mW, argon laser) was employed in order to excite fluorescein and phycoerythrin. The electronic compensation of the machine for FL1-FL2 and FL2-FL1 was adjusted for each combination of MAb to optimise the two-colour analyses. Linear amplification was used for the physical parameters (FSC, SSC) and logarithmic amplification used for the fluorescence parameters (FL1-green, FL2-red).

Between $5-10 \times 10^3$ cells were collected per sample and red and green fluorescence analyses were performed on viable resting lymphocytes and blast cells, discriminated from dead cells and alveolar macrophages on the basis of their FSC versus SSC light scatter.

Table 4.1. Monoclonal antibodies (MAbs) and immunoconjugates employed for dual-colour flow cytometric analyses

MAbs	ISOTYPE	ANTIGEN IDENTIFIED	REFERENCE
BIOTINYLATED			
SBU-T4	IgG _{2a}	Ovine CD4	Maddox <i>et al</i> , 1985b
SBU-T8	IgG _{2a}	Ovine CD8	Maddox <i>et al</i> , 1985b
CC15	IgG _{2b}	Ovine WC1 ($\gamma\delta$ T cells)	Mackay <i>et al</i> , 1991
UNCONJUGATED			
SW73.2	IgG _{2b}	Total ovine MHC Class II β	Hopkins <i>et al</i> , 1986
VPM54	IgG ₁	Ovine MHC Class II α (DR)	Dutia <i>et al</i> , 1990a; 1990b
VPM36	IgG ₁	Ovine MHC Class II α (DQ)	Dutia <i>et al</i> , 1990a; 1990b
IL-A111	IgG ₁	Ovine IL-2R (α chain)	Naessens <i>et al</i> , 1992
IMMUNOCONJUGATES			
Rabbit antimouse IgG ₁			
Rabbit anti-rat IgG _{2b}			
Phycoerythrin labelled streptavidin			

Table 4.2. Optimal working dilutions for monoclonal antibodies and conjugates for dual colour flow cytometry

MAbs	DILUTION
NMS	1:1000
NRS	1:500
SW73.2	1:2
VPM54	1:2
VPM36	1:2
IL-111A	1:2000
SBU-T4 biotin	1:800
SBU-T8 biotin	1:800
CC15- biotin	1:100
PE-SA complex	1:200
Rabbit anti-mouse IgG₁	1:250
Rabbit anti-rat IgG_{2b}	1:1000

4.3.6 STATISTICAL ANALYSES

Results obtained from the double staining were analysed in two different ways: firstly, by setting quadrants by reference to a relevant negative control (as explained in Chapter 2) and then calculating the percentage of double positively labelled cells and secondly, by performing curve subtraction analyses. As data were non normally distributed, BALF and PB sample comparisons between MVV-infected and control sheep were made using the Mann-Whitney non parametric test, with a significance level of 95%.

4.4. RESULTS

4.4.1 LYMPHOCYTIC PHENOTYPES IN BALF AND PB

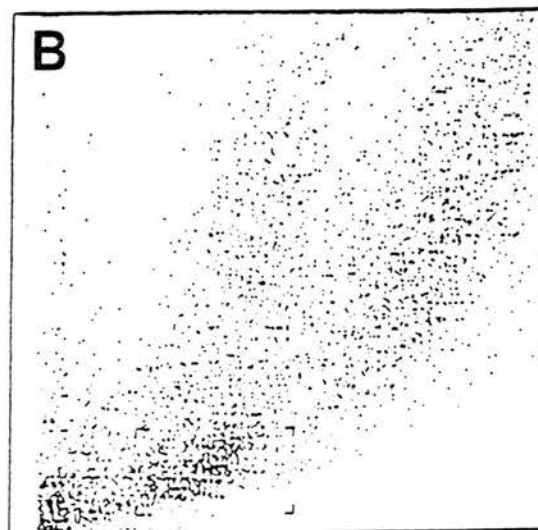
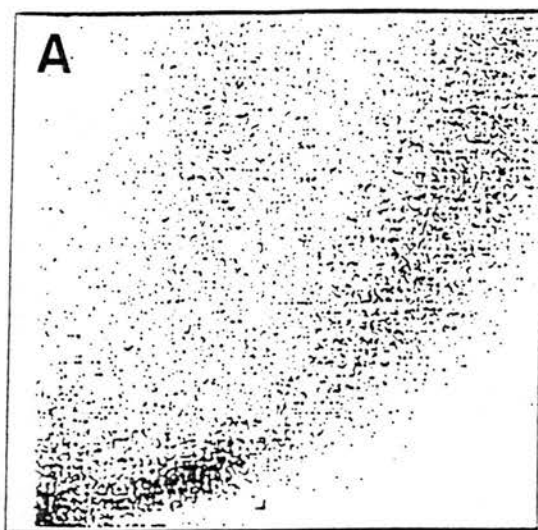
As reported in previous studies (Lujan *et al*, 1993), the most consistent findings in the BALF lymphocyte subsets from MVV-infected animals were a decrease in the percentage of CD4⁺ T cells [control= 39.1 (32.5-50.8); MVV= 23.4 (11.4-40.8)], an increase in the percentage of CD8⁺ T cells [control= 17.3 (9.8-35.6); MVV= 29.6 (12.1-74.9)] and a significant inversion ($p < 0.05$) of the CD4⁺/CD8⁺ ratio [control= 2.9 (0.9-3.9); MVV= 1.0 (0.2-1.3)] (results expressed as median and range of percentages of positively labelled cells). No changes were observed in the percentage of $\gamma\delta$ T cell population. No significant differences in PB lymphocyte subsets were observed between the two groups of animals.

4.4.2 BLAST TRANSFORMATION IN LYMPHOCYTES FROM BALF AND PB

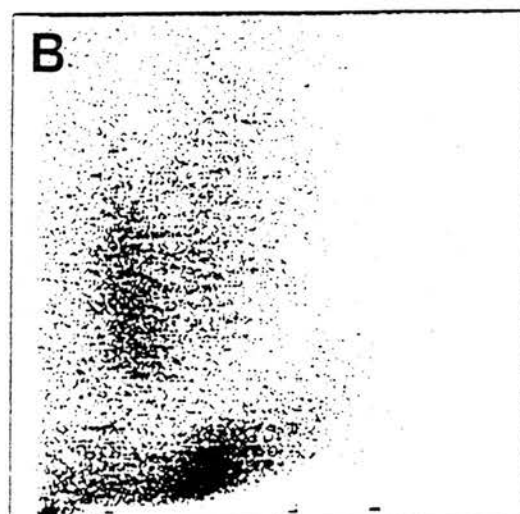
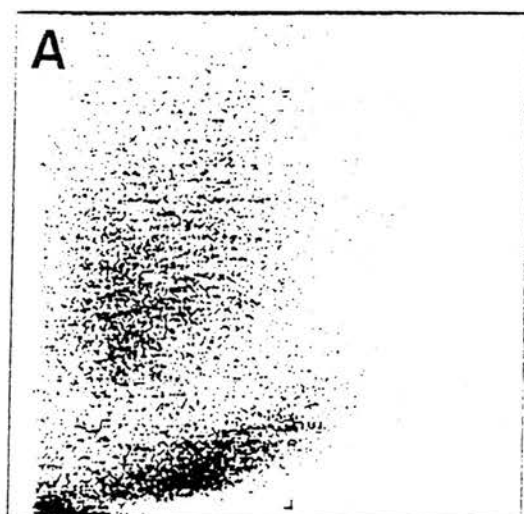
In both BALF and PB, lymphocytes from control and MVV-infected animals showed similar distribution on the FSC/SSC dot plot (Fig. 4.1).

Figure 4.1. Light scatter profiles of BALF and PB from a control (A) and a MVV-infected (B) animal. The gate shows the lymphocyte population that is present in the same location for both groups of animals. No blast transformation is observed.

BALF



PB



4.4.3 MHC CLASS II EXPRESSION IN BALF

4.4.3.1 CD4⁺ T LYMPHOCYTES

The coexpression of CD4 and MHC Class II molecules on BALF lymphocytes is shown in Table 4.3 and Figure 4.2, respectively.

MVV-infected animals demonstrated a significant ($p < 0.05$) increase in the percentage of CD4⁺ T cells expressing all the MHC Class II MAbs employed, namely SW73.2, VPM54 (DR) and VPM36 (DQ), both by quadrant and by curve subtraction analyses.

4.4.3.2 CD8⁺ T LYMPHOCYTES

Table 4.4 and Fig. 4.3. show the coexpression of CD8 and MHC Class II molecules on T cells. In the CD8⁺ T cell subpopulation of MVV-infected animals there was a significant increase ($p < 0.05$) in the percentage of cells expressing SW73.2 and VPM54 (DR). The percentage of CD8⁺ T cells positive for VPM36 (DQ) was increased in the MVV-infected animals, although this increase was not significant. Results obtained by quadrant statistics were consistent with those obtained by curve subtraction analyses.

4.4.3.3 $\gamma\delta$ T LYMPHOCYTES

The coexpression of $\gamma\delta$ and MHC Class II molecules on T cells is presented in Table 4.5 and Fig. 4.4, respectively. There were very few $\gamma\delta$ -TCR lymphocytes in the BALF of both control and MVV-infected animals [control= 5.3 (2.8-11.1); MVV= 3.0 (1.5-9.9)] (results expressed as median and range of percentages of positively labelled cells in lymphocyte gate). However, most of the $\gamma\delta$ -TCR lymphocytes in both groups of animals expressed the whole range of the MHC Class II molecules. $\gamma\delta$ -TCR BALF lymphocytes from the MVV-infected group expressed a higher density of MHC Class II molecules as compared with controls, although this difference was not significant.

Table 4.3. Coexpression of MHC Class II molecules or IL-2R and CD4 on T lymphocytes in bronchoalveolar lavage fluid from control and MVV- infected sheep

	CD4/SW73.2	CD4/VP54	CD4/VP36	CD4/IL-2R
(a) CONTROL n=7	42.9 (10.7-57.2)	19.2 (8.1-59.3)	12.8 (3.1-30.4)	29.6 (25.3-37.8)
(b)	32.1 (10.8-60.0)	12.7 (10.1-23.9)	14.0 (3.4-23.5)	16.3 (4.4-35.1)
(a) MAEDI n=7	81.4* (50.7-90.7)	50.0* (27.0-84.1)	52.6* (20.2-88.1)	25.9 (12.5-96.8)
(b)	76.4* (17.5-87.2)	53.9* (17.5-82.5)	55.1* (17.3-79.2)	17.6 (17.3-37.3)

Results are expressed as (a) median of percentages of double positively labelled cells calculated by quadrant statistics, and (b) as median of percentages from curve subtraction. Ranges in parentheses.

*Significantly different from controls at $p < 0.05$ (Mann-Whitney non parametric rank test)

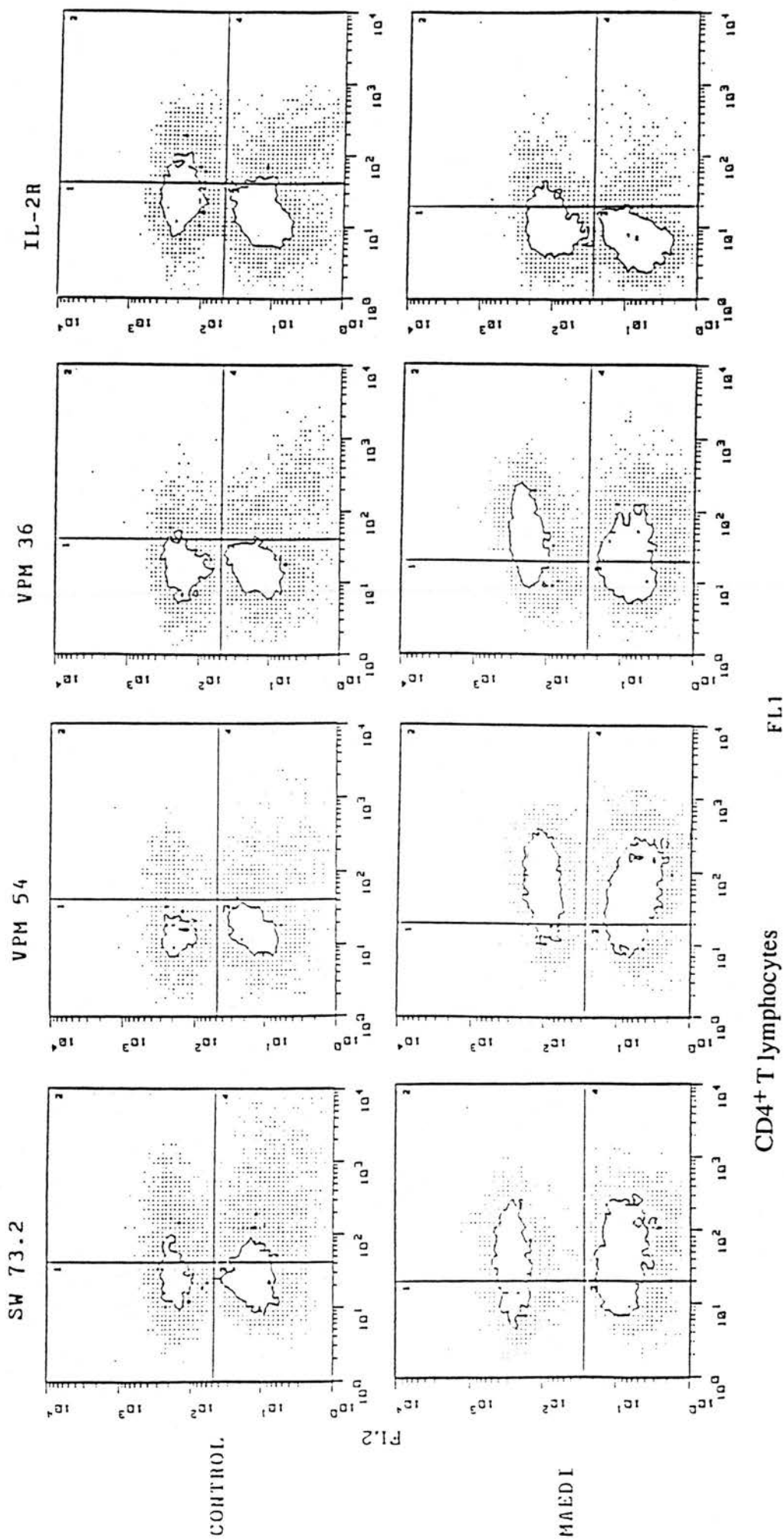


Figure 4.2. Contour maps of dual stained CD4⁺ lymphocytes from BALF from a control and a MVV-infected animal. FL-1 (green fluorescence) on the abscissa and FL-2 (red fluorescence) on the ordinate. FL-2 distinguishes CD4⁺, and FL-1 distinguishes MHC Class II and IL-2R expression. An increase in MHC Class II molecules is observed for SW73.2 (pan-Class II), VPM54(DR) and VPM36 (DQ), while there is no increase in IL-2R expression.

Table 4.4. Coexpression of MHC Class II molecules or IL-2R and CD8 on T lymphocytes in bronchoalveolar lavage fluid from control and MVV- infected sheep

	CD8/SW73.2	CD8/VPM54	CD8/VPM36	CD8/IL-2R
(a) CONTROL n=7	55.2 (23.4-75.6)	26.7 (15.9-66.8)	15.5 (7.5-61.1)	7.5 (12.8-39.5)
(b)	20.6 (5.5-52.5)	27.3 (6.3-41.1)	17.2 (4.6-31.4)	21.1 (8.8-33.9)
(a) MAEDI n=7	89.2* (72.1-100)	69.4* (41.6-90.2)	36.9 (9.7-78.3)	13.4 (1.5-77.3)
(b)	71.6* (9.4-85.2)	60.3* (6.3-84.7)	48.6 (5.5-64.9)	30.6 (2.3-36.9)

Results are expressed as (a) median of percentages of double positively labelled cells calculated by quadrant statistics and (b) as median of percentages from curve subtraction. Ranges in parentheses.

*Significantly different from controls at $p < 0.05$ (Mann-Whitney non parametric rank test)

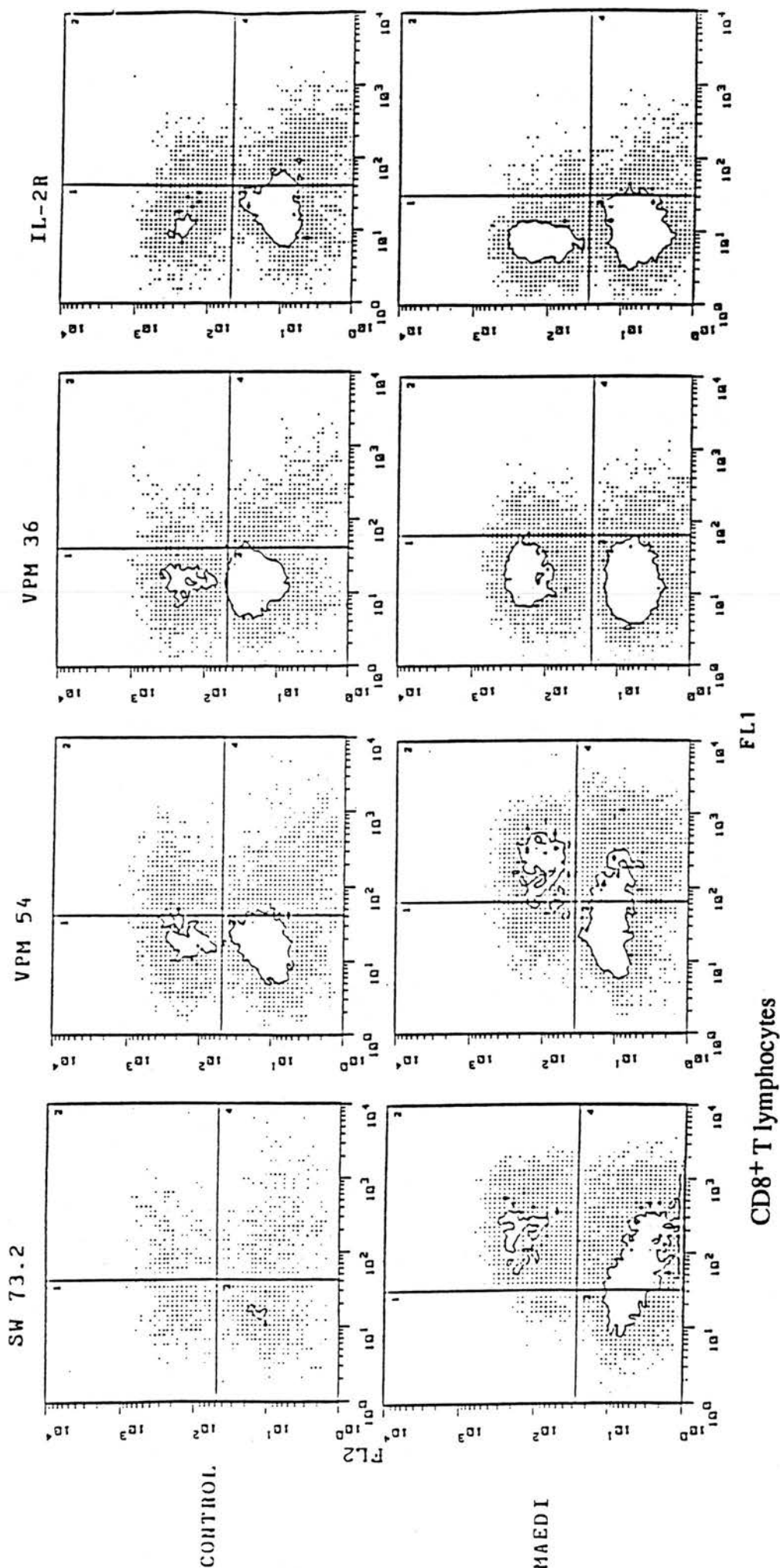


Figure 4.3. Contour maps of dual stained $CD8^+$ lymphocytes from BALF from a control and a MVV-infected animal. FL-1 (green fluorescence) on the abscissa and FL-2 (red fluorescence) on the ordinate. FL-2 distinguishes $CD8^+$, and FL-1 distinguishes MHC Class II and IL-2R expression. An increase in MHC Class II molecules is observed for SW73.2 (pan-Class II) and VPM54(DR), while there is no increase in VPM36 (DQ) or IL-2R expression.

Table 4.5. Coexpression of MHC Class II or IL-2R molecules and $\gamma\delta$ on T lymphocytes in bronchoalveolar lavage fluid from control and MVV-infected sheep

	$\gamma\delta$ /SW73.2	$\gamma\delta$ /VPM54	$\gamma\delta$ /VPM36	$\gamma\delta$ /IL-2R
CONTROL n=7	56.0 (14.1-93.6)	66.1 (32.6-96.7)	86.1 (28.8-96.7)	81.0 (28.8-96.7)
MAEDI n=7	87.2 (63.8-97.9)	94.5 (85.3-97.9)	93.5 (64.2-99.2)	92.7 (56.5-97.8)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

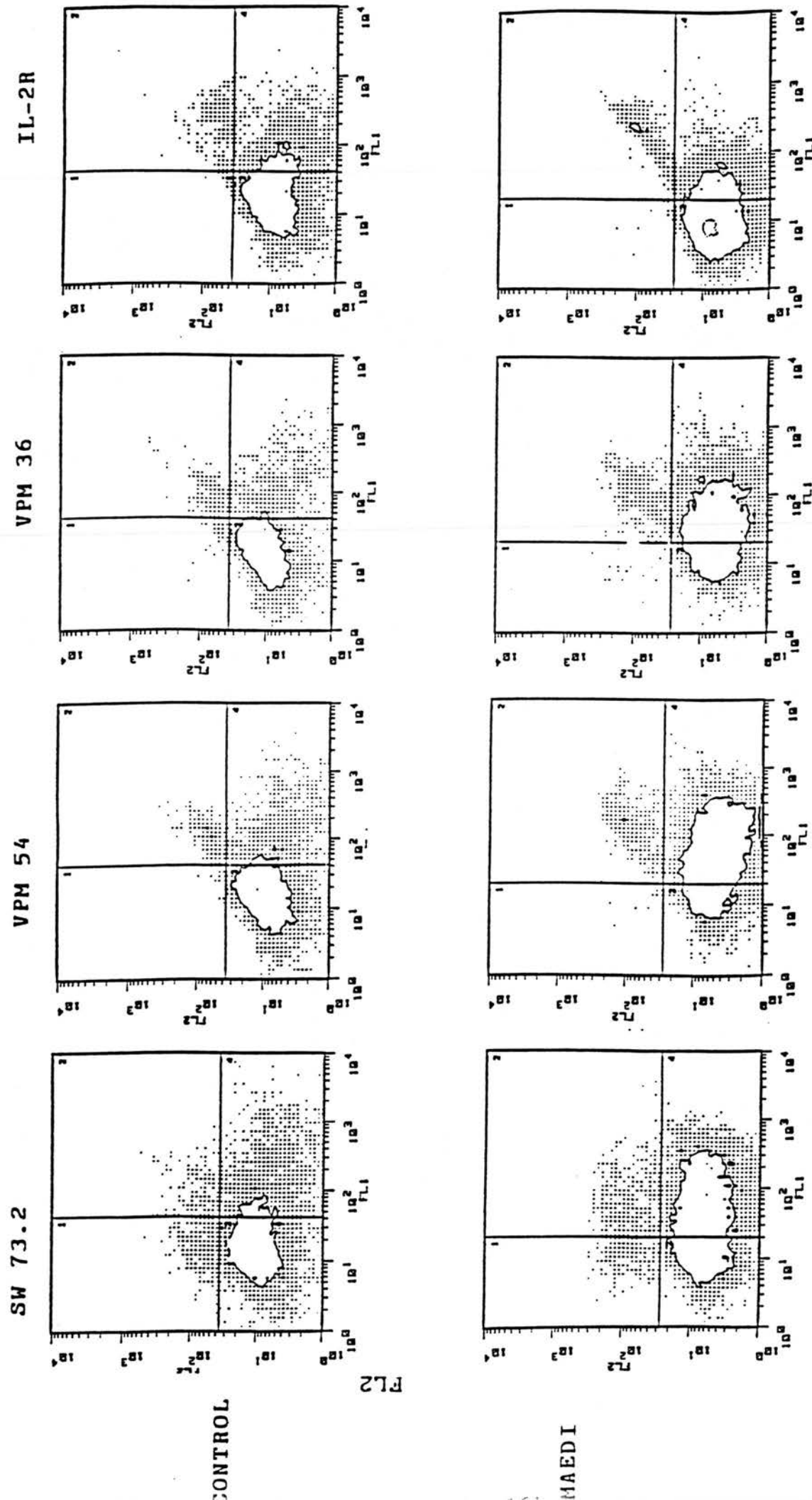


Figure 4.4. Contour maps of dual stained $\gamma\delta$ T-lymphocytes from BALF from a control and a MVV-infected animal. FL-1 (green fluorescence) on the abscissa and FL-2 (red fluorescence) on the ordinate. FL-2 distinguishes $\gamma\delta$ T cells, and FL-1 distinguishes MHC Class II and IL-2R expression. A non significant increase in MHC Class II molecules is observed for SW73.2 (pan-Class II), VPM54 (DR) and VPM36 (DQ), while there is no increase in IL-2R expression.

4.4.3.4 IL-2R EXPRESSION

Tables 4.3, 4.4 and 4.5 and Figs. 4.2, 4.3 and 4.4 show the expression of IL-2R molecules by BALF lymphocyte subsets. There were no significant differences in the expression of IL-2R by any of the three T cell subsets between control and MVV-infected animals as confirmed both by quadrant statistic and by curve subtraction analyses.

The mean channel number of IL-2R expression on BALF T lymphocytes was also studied, and it did not revealed any significant differences between the control and the MVV-infected group (Appendix I.4.1)

4.4.4 PB LYMPHOCYTE PHENOTYPING

No significant differences between the two groups of animals were observed for the expression of MHC Class II or IL-2R by any of the three T cell subsets in PB.

Results are shown in Tables 4.6, 4.7 and 4.8 and in Figs. 4.5, 4.6 and 4.7.

Table 4.6. Coexpression of MHC Class II or IL-2R molecules and CD4 on T lymphocytes in peripheral blood from control and MVV-infected sheep

	CD4/VPM54	CD4/ VPM36	CD4/IL-2R
CONTROL n=5	24.6 (16.5-39.5)	7.9 (6.6-18.6)	27.67 (23.5-33.1)
MAEDI n=8	33.3 (3.9-67.1)	14.5 (6.2-37.6)	37.8 (20.4-48.8)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses.

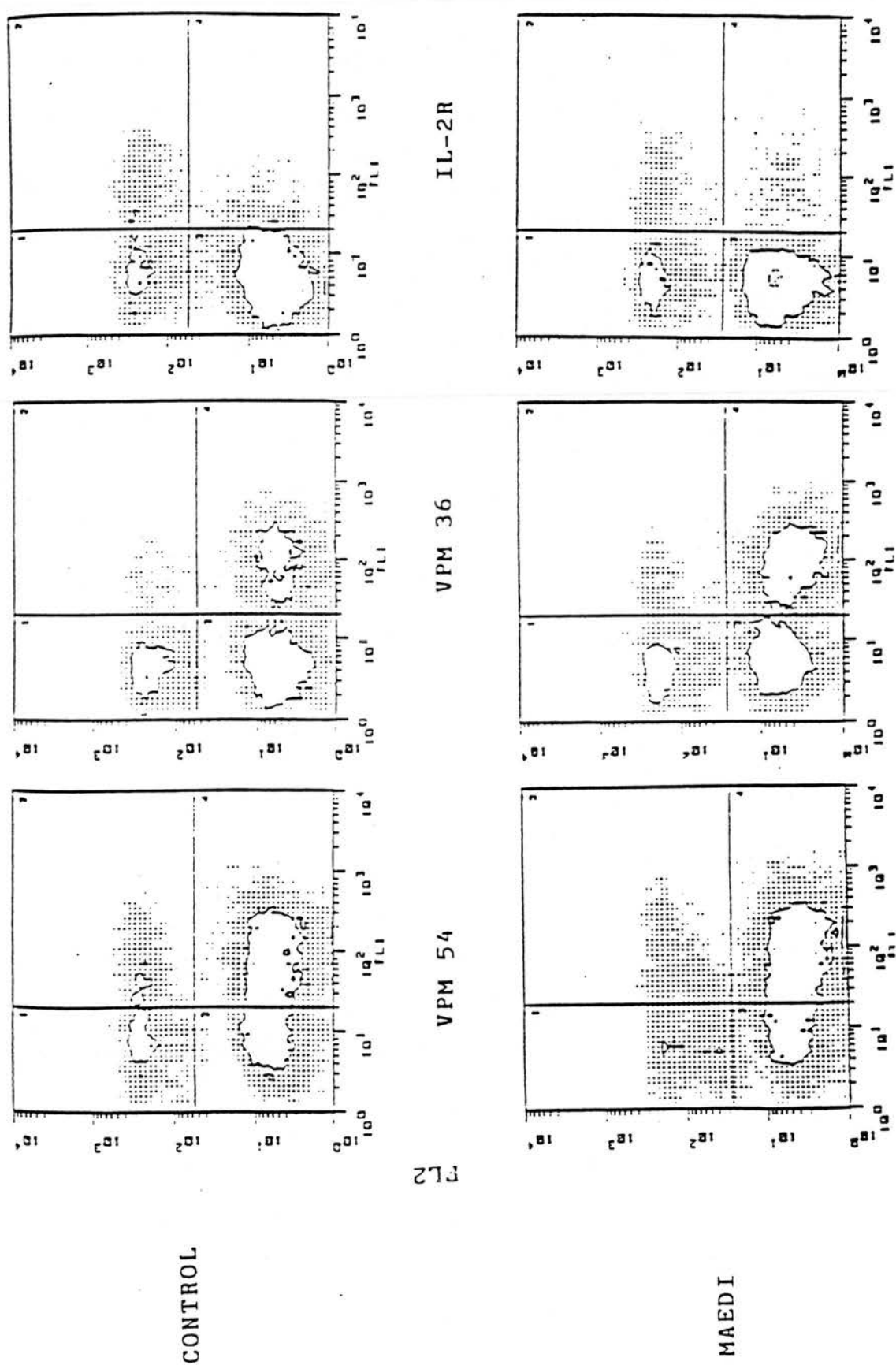


Figure 4.5. Contour maps of dual stained CD4⁺ PB lymphocytes from a control and a MVV-infected animal. FL-1 (green fluorescence) on the abscissa and FL-2 (red fluorescence) on the ordinate. FL-2 distinguishes CD4⁺, and FL-1 distinguishes MHC Class II and IL-2R expression. No differences in the expression of MHC Class II molecules or IL-2R are observed between the control and the MVV-infected animal.

Table 4.7. Coexpression of MHC Class II or IL-2R molecules and CD8 on T lymphocytes in peripheral blood from control and MVV-infected sheep

	CD8/VPM54	CD8/VPM36	CD8/IL-2R
CONTROL n=5	42.9 (14.1-52.0)	8.5 (4.7-22.3)	1.2 (1.1-1.3)
MAEDI n=8	56.2 (25.6-88.6)	30.3 (5.5-64.9)	3.7 (1.1-9.3)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses.

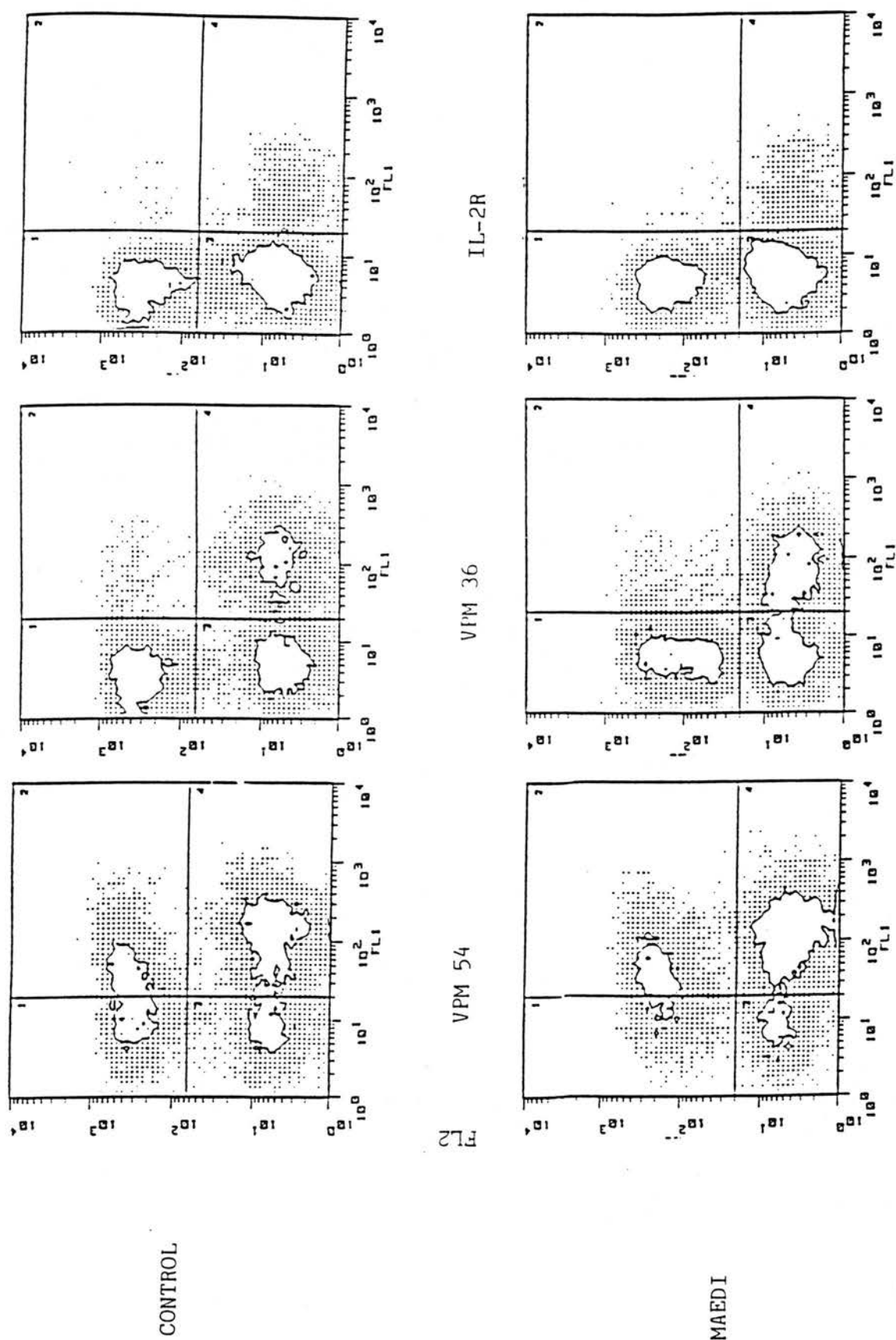


Figure 4.6. Contour maps of dual stained $CD8^+$ PB lymphocytes from a control and a MVV-infected animal. FL-1 (green fluorescence) on the abscissa and FL-2 (red fluorescence) on the ordinate. FL-2 distinguishes $CD8^+$, and FL-1 distinguishes MHC Class II and IL-2R expression. No differences in the expression of MHC Class II molecules or IL-2R are observed between the control and the MVV-infected animal.

Table 4.8. Coexpression of MHC Class II or IL-2R molecules and $\gamma\delta$ on T lymphocytes in peripheral blood from control and MVV-infected sheep

	$\gamma\delta$ /VPM54	$\gamma\delta$ /VPM36	$\gamma\delta$ /IL-2R
CONTROL n=5	17.1 (5.1-46.2)	11.3 (5.6-27.1)	18.1 (11.5-26.7)
MAEDI n=8	35.5 (21.4-57.5)	15.4 (8.4-57.5)	22.9 (13.6-35.3)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses.

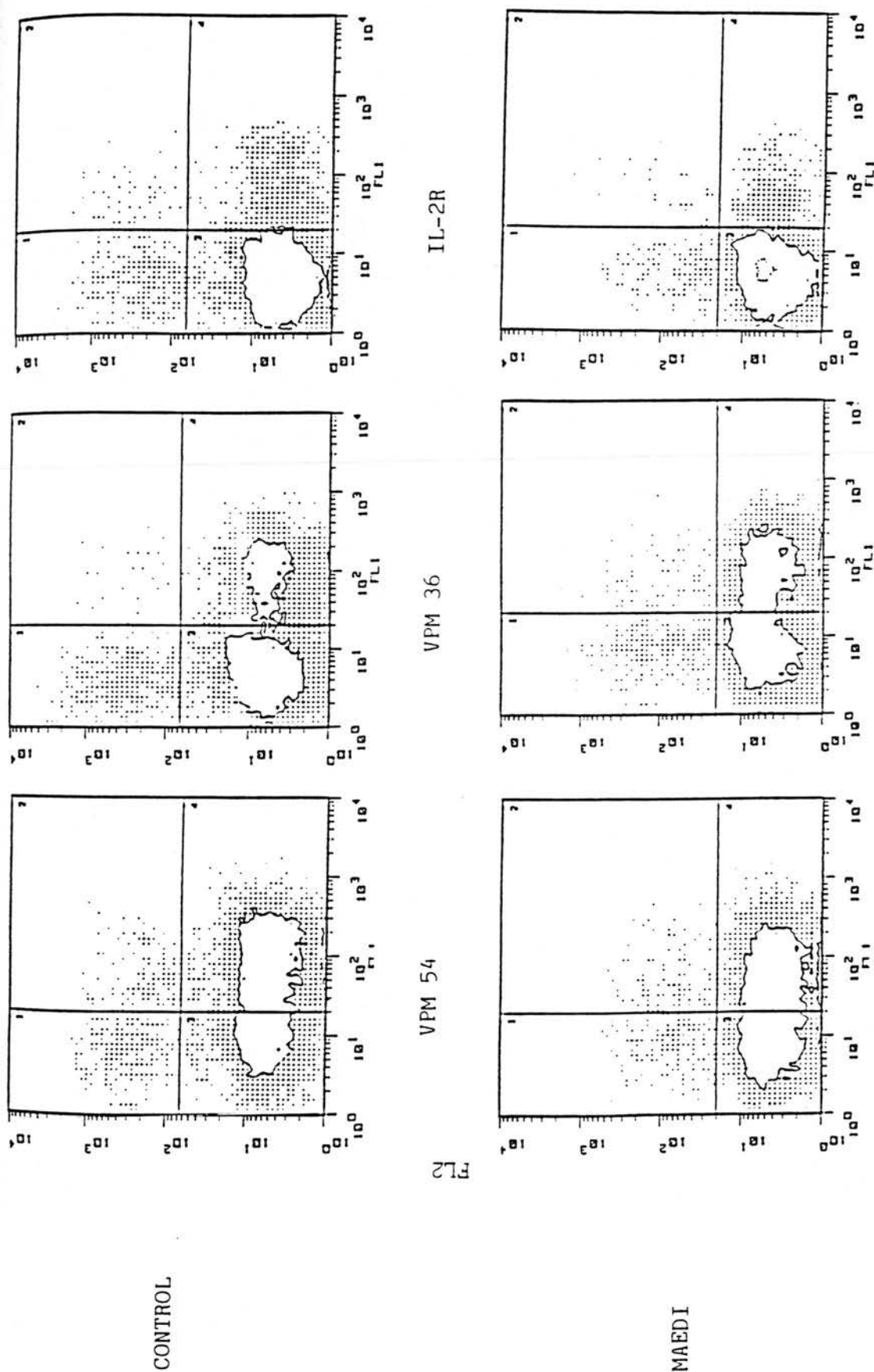


Figure 4.7. Contour maps of dual stained $\gamma\delta$ PB lymphocytes from a control and a MVV-infected animal. FL-1 (green fluorescence) on the abscissa and FL-2 (red fluorescence) on the ordinate. FL-2 distinguishes $\gamma\delta$ T cells, and FL-1 distinguishes MHC Class II and IL-2R expression. No differences in the expression of MHC Class II molecules are observed for any of the MAb used: VPM54(DR), VPM36 (DQ) and IL-2R between the control and the MVV-infected animal.

4.5. DISCUSSION

In this study lymphocyte subsets in BALF and PB for control and MVV-infected animals were of similar phenotype to those previously described in Chapter 3. The measurement of three parameters generally considered as markers of activation, i.e. blast transformation, expression of MHC Class II molecules and expression of IL-2R, permitted investigation of the stage of activation of the BALF and PB T lymphocytes of naturally MVV-infected sheep.

The availability of the MAbs VPM54 and VPM36 (Dutia *et al*, 1990a;1990b), homologous to the human DR and DQ respectively, permitted the study of the differential expression of DR and DQ locus products on BALF T cell subpopulations. Marked differences in expression of MHC Class II antigens with respect to lymphocytes in other body compartments from sheep were found (Table 4.9). While in adult peripheral blood and efferent lymph MHC Class II molecules are mainly DR, with very little DQ expression (Dutia *et al*, 1993), in BALF from both adult control and MVV-infected animals a considerable proportion of CD4⁺, CD8⁺ and $\gamma\delta$ T lymphocytes also express DQ molecules. In adult sheep afferent lymph, the majority of all T cells express both DQ and DR molecules, with a predominance of DR molecules, and in the lymph node over 50% of the cells express DR and 30% are DQ⁺ (Dutia *et al*, 1993). No previous quantification of the different locus products on T cells from the ovine lung has been reported.

In man, specific functions for the different isotypes of MHC Class II have been proposed. Hence, while DR may present antigen specifically to T_H cells, regulating the immune response, DQ molecules may be responsible for the presentation of antigen to T_s cells (Nieda *et al*, 1988) and are involved in the control of immune suppression (Hirayama *et al*, 1987; Oliveira and Mitchison, 1989). In this induction of suppression, DQ molecules might contribute directly to activation of CD8⁺ T cells, or

Table 4.9. Percentage of T lymphocytes expressing MHC Class II molecules in different body compartments of sheep. Data for peripheral blood mononuclear cells (PBMC), efferent, afferent lymph and lymph node were determined by Dutia *et al* (1993). Data for BALF were determined in this study.

	PBMC	EFFERENT LYMPH	AFFERENT LYMPH	LYMPH NODE	BALF
CD4⁺ T cells					
<i>DQ</i>	3.5 ± 2.8	7.0 ± 2.0	80.1 ± 3.6	33.6 ± 4.6	23.7 ± 18.3
<i>DR</i>	57.1 ± 8.3	30.0 ± 3.0	89.1 ± 8.3	51.6 ± 5.2	13.8 ± 8.6
CD8⁺ T cells					
<i>DQ</i>	6.4 ± 3.9	13.0 ± 2.0	73.8 ± 4.2	30.3 ± 3.2	33.0 ± 18.3
<i>DR</i>	51.4 ± 7.3	38.4 ± 4.0	79.8 ± 6.8	56.5 ± 6.3	25.1 ± 19.0
γδ⁺ T cells					
<i>DQ</i>	<1	6.0 ± 3.0	60.6 ± 6.2	48.3 ± 4.7	65.8 ± 26.7
<i>DR</i>	35.3 ± 9.3	19.0 ± 2.0	78.7 ± 4.8	62.3 ± 8.2	80.9 ± 17.3

indirectly through the activation of CD4⁺ suppressor-inducer T cells (Yachie *et al*, 1982; Dorf and Benacerraf, 1985). It has also been suggested that DQ molecules might play a major role in predisposition to autoimmune diseases such as type I (insulin-dependent) diabetes mellitus (Todd *et al*, 1987), thyrotoxicosis (Foulis, 1986) and primary biliary cirrhosis (Ballardini *et al*, 1984). MHC Class II-DR antigens on T cells may mediate signal transduction (Odum *et al*, 1991). Enhanced expression of DR⁺ lymphocytes has been found on peripheral blood CD8⁺ T cells from patients with AIDS and with the pre-AIDS syndrome (Engleman *et al*, 1980) and in patients with idiopathic myositis (Davidson *et al*, 1985).

Similarly to man, the differential expression of DR and DQ locus products of ovine BALF T lymphocytes might also be related to the existence of functional differences between them. Experiments to investigate their possible different roles are currently in progress (Hopkins *et al*, 1993b).

Increased cell size and complexity, produced as a consequence of antigenic stimulation, are regarded as early parameters of activation (Goodman, 1983; Samelson, 1989). These changes, however, were not found in MVV-infected sheep. This lack of blast transformation in BALF and PB lymphocytes of MVV-infected animals could be the first indicator of an impaired lymphocyte activation.

In contrast, MHC Class II molecules, which are also regarded as activation markers for T lymphocytes (Cotner *et al*, 1983), were increased in BALF T lymphocytes of MVV-infected animals. Only CD4⁺ and CD8⁺ T lymphocytes showed upregulation of MHC Class II molecules, while no significant changes were observed in the $\gamma\delta$ T population. CD4⁺ and CD8⁺ T lymphocytes were also the main T cell subsets that had shown phenotypic changes in previous studies (Cordier *et al*, 1992; Lujan *et al*, 1993).

The existence of a proportion of ovine resting T-lymphocytes bearing MHC Class II antigens has been described (Dutia *et al*, 1993; Hopkins *et al*, 1993b; Burrells and Sutherland, 1994). Our results in control animals were consistent with these reports. The constitutive expression of MHC Class II antigens by ovine T cells suggests an important role for these molecules in normal T cell function.

In MVV-infected animals, the upregulated expression of MHC Class II antigens by BALF T lymphocytes is concordant with human interstitial lung diseases, including pulmonary sarcoidosis (Hunninghake and Crystal, 1981; Mornex *et al*, 1985) and hypersensitivity pneumonitis (Mornex *et al*, 1984).

Hopkins and others (1993b) showed that the expression of MHC Class II-DQ molecules by sheep efferent lymph T cells was the result of recent activation. Presumably, ovine T cells from other body compartments will also require a previous process of activation prior to expression of DQ locus products. If this assumption is true, then upregulated expression of DQ locus products by BALF T lymphocytes from MVV-infected animals indicates an ongoing process of activation within the lung. Accumulation of activated lymphocytes in the lung would contribute to the interstitial lung disease through the production of a network of inflammatory cytokines. These cytokines may increase the expression of MHC Class II antigens by T cells (Steinman *et al*, 1980) and by alveolar macrophages. Increased expression of MHC Class II molecules on BALF macrophages of naturally MVV-infected sheep had been observed (Chapter 3). It is known that ovine MHC Class II antigens act as a component of a cellular receptor for MVV (Dalziel *et al*, 1991). Hence, enhanced MHC Class II molecules expression by alveolar macrophages would amplify the number of potential MVV-infected alveolar macrophages and could facilitate spread and persistence of the virus.

The major function of MHC Class II molecules is to present processed antigen to T cells, thus playing a central role in the induction of the immune response. The

functional role of these antigens on 'activated' T cells remains largely unknown, although the magnitude of T cell responses seems to be related to the level of expression of MHC Class II antigen (Matis *et al*, 1983). Hence, quantitative variation in MHC Class II expression is associated with immunodeficiency, and could explain human immunological diseases such as the lymphomatous form of Hodgkin's disease, lepromatous leprosy or sarcoidosis (Janeway *et al*, 1984). Also, as MHC Class II antigen is the natural ligand for CD4, its induction on activated T cells may play a role on regulating certain CD4 functions (Odum *et al*, 1991), such as cell adhesion (Doyle and Strominger, 1987).

In sheep, as in man and mice, expression of MHC Class II on T cells is involved in the presentation of soluble antigen *in vivo*. However, apart from this presentational function, a regulatory role is also suggested (Hopkins *et al*, 1993b).

Impaired lymphocyte activation is also suggested by the finding that levels of IL-2R expression on BALF T lymphocytes from infected sheep were not elevated, despite using a highly sensitive assay for detection of IL-2R (Zola *et al*, 1990; Santos *et al*, 1991). IL-2R is expressed at low levels on normal human BALF T lymphocytes (Pinkston *et al*, 1983; Becker *et al*, 1990) and the increase of this receptor is considered as a parameter of lymphocyte activation (Zola *et al*, 1991). However, disorders of IL-2R expression have been reported in primary and acquired immunodeficiency and rheumatic diseases (Prince *et al*, 1984; Waldmann *et al*, 1985; Greene *et al*, 1986b; Campen *et al*, 1988; Mahalingam *et al*, 1993) and in blood of patients with insulin-dependent diabetes mellitus (Kontiainen *et al*, 1991). In lymph nodes of lentiviral-infected sheep, both decreased IL-2 production (Ellis and DeMartini, 1985a) and lack of IL-2R expression by the CD8⁺ T subpopulation (Bird *et al*, 1993) have been observed.

The fact that elevated MHC Class II expression on BALF T lymphocytes from MVV-infected sheep was not coincident with a similar elevation in IL-2R expression suggests that different forms of activation are possible, each cell being characterised by particular phenotypes. Alternatively, the upregulation of MHC Class II molecules on T cells of MVV-infected animals may not be simply a consequence of a viral-induced T cell activation. Discrepancy between Class II and IL-2R expression has also been found in some inflammatory lesions in synovial tissues (Burmester *et al*, 1984), in feline immunodeficiency virus infection (FIV) (Rideout *et al*, 1992) and in Sjogren's syndrome (Jonhson *et al*, 1987), an autoimmune disease of man which has pulmonary histopathologic similarities to MVV infection.

The increased MHC Class II antigen expression in BALF T lymphocytes from MVV-infected animals has two possible explanations. Firstly, an upregulation induced by cytokines from activated T cells, such as γ -IFN (Nash *et al*, 1992; Hopkins *et al*, 1993b) and, in the case of MVV-infected animals, to the specific lentivirus-induced interferon (LV-IFN) (Kennedy *et al*, 1985). Hence, the higher levels of MHC Class II molecules on BALF T lymphocytes from MVV-infected animals might reflect a cytokine-mediated immune dysfunction. Secondly, the persistent viral infection of alveolar macrophages could cause chronic stimulation of BALF T lymphocytes, which would then maintain enhanced expression of MHC Class II antigens on their surface.

Several possible explanations can be proposed for the absence of a concomitantly increased expression of IL-2R in BALF T lymphocytes from MVV-infected animal;

Firstly, lymphocytes could be going through an impaired process of activation.

Secondly, this absence could be due to decreased levels of IL-2 in BALF, as IL-2 is a preliminary requisite for IL-2R expression (Williams *et al*, 1985). Decreased

levels in IL-2 activity have been found in mononuclear leukocytes culture supernatants derived from the posterior mediastinal lymph nodes of MVV-infected sheep (Ellis and DeMartini, 1985a). The levels of IL-2 present in BALF were not investigated in this study.

Thirdly, a decline of IL-2R expression could also be possible, as *in vitro* studies have shown that the expression of this receptor on activated cells is transient after antigenic stimulation (Depper *et al*, 1984), this expression being restored by reexposure to initial stimuli (Cantrell and Smith, 1983).

Fourthly, other lymphokines present in the PELF, such as an IL-2 inhibitory factor or a suppressor factor could interfere with the production of IL-2 (Neng Lai *et al*, 1989) and the expression of IL-2R.

Finally, viral infection and/or subsequent activation of macrophages could lead to decreased IL-2R expression, as occurs in FIV infection (Lawrence *et al*, 1992).

To explain the increased expression of MHC Class II molecules by BALF T lymphocytes and the lack of a similar elevation in IL-2R expression we favour the hypothesis that BALF T lymphocytes from MVV-infected sheep are undergoing an impaired process of lymphocyte activation. Some aspects of the pathogenesis of MVV infection, especially those regarding viral persistence, would lead to disruption of the immune system. As a consequence, some cellular immune responses may be amplified (i.e. upregulated expression of MHC Class II antigens), while others (i.e. IL-2R expression) may be suppressed. The increased proportion of BALF CD8⁺ T lymphocytes could also contribute to a defect in IL-2R expression, as CD8⁺ T cells are thought to contain suppressor as well as cytotoxic lymphocytes (Takata *et al*, 1990). In addition, viral infection of alveolar macrophages could give rise to a cytokine-mediated dysregulation which could contribute to recruitment of increased numbers of lymphocytes to the alveoli, leading to inflammation and lung pathology.

Further studies are required to investigate if this anomalous pattern of lymphocyte activation observed during the course of the natural disease is maintained under *in vitro* conditions.

Finally, the fact that no significant changes in MHC Class II molecules expression in PBL between control and MVV-infected animals were found in this study, further suggests a compartmentalisation of the immune response to the lung during the course of MVV infection, as was discussed in Chapter 3.

CHAPTER 5: *IN VITRO* BALF AND PB LYMPHOCYTE RESPONSE TO MITOGEN-STIMULATION DURING NATURAL MVV INFECTION

5.1. SUMMARY

To investigate the effects of MVV infection on cell-mediated immunity, the *in vitro* response of BALF and PB lymphocytes to exogenous mitogen was investigated. BALF and PB lymphocytes from control (n=9) and MVV-infected (n=7) animals were cultured for 3 days in the presence and absence of concanavalin A (Con A). Lymphocyte expression of an early activation marker, the IL-2R antigen, a parameter of lymphocyte activation, was quantified by dual-colour flow cytometry using the bovine anti-IL-2R MAb IL-A111. IL-2R expression by lymphocytes in BALF and PB from control and MVV-infected animals, with and without Con A stimulation, were compared.

In the absence of Con A stimulation, IL-2R expression on cultured BALF CD8⁺ and $\gamma\delta$ T cells was significantly ($p < 0.05$) lower for MVV-infected animals than for controls. After Con A stimulation, expression of IL-2R on BALF CD4⁺ lymphocytes from MVV-infected animals remained significantly ($p < 0.05$) lower than that of cells from controls. Comparisons within group showed that the control group, after Con A-stimulation, had significantly increased IL-2R expression by all the T cell subsets, namely CD4⁺ ($p < 0.001$), CD8⁺ ($p < 0.001$) and $\gamma\delta$ T cells ($p < 0.05$). In the MVV-infected group, this increase was significant ($p < 0.05$) for CD4⁺ and CD8⁺ T cells, but not for $\gamma\delta$ T cells.

In vitro mitogen stimulation of PB T lymphocytes from both control and MVV-infected animals induced a significant elevation in IL-2R expression for all T cell subsets when compared to cultured unstimulated control cells. However, there was considerable heterogeneity in the response to Con A of PB T cells from both groups of animals. The expression of IL-2R followed a different pattern to that of

BALF lymphocytes, unstimulated $\gamma\delta$ T cells from MVV-infected animals having significantly ($p < 0.05$) higher IL-2R expression than those of controls. Cultured unstimulated CD8⁺ T cells from MVV-infected animals had significantly ($p < 0.05$) lower IL-2R expression than cultured unstimulated CD8⁺ cells from controls.

From these studies it can be concluded that the BALF T lymphocyte immune dysfunction observed during natural MVV infection, characterised by impaired IL-2R expression, is maintained under *in vitro* conditions.

5.2. INTRODUCTION

The previous investigation of the state of activation of BALF and PB T lymphocytes from naturally MVV-infected animals showed an anomalous pattern of BALF T lymphocyte activation. Hence, while BALF T lymphocytes from MVV-infected animals expressed MHC Class II molecules strongly on their surface, one of the parameters suggesting a process of activation, increased surface IL-2R expression, another main index for lymphocyte activation, was lacking.

This study was developed to assess whether the defective lymphocyte activation observed in the natural disease was maintained under *in vitro* conditions.

5.2.1 THE STUDY OF LYMPHOCYTE TRANSFORMATION BY FLOW CYTOMETRY

Both mitogen- and antigen-induced lymphocyte transformations have become widely used tools in experimental studies and for practical clinical tests. The discovery (Nowell, 1960) that phytohemagglutinin (PHA), a lectin extracted from kidney beans, transformed small lymphocytes into proliferating lymphoblasts in tissue culture formed the basis of this technique. Today, T-cell mitogens commonly used together with PHA are the lectins concanavalin A (Con A) (obtained from the jack bean), and pokeweed mitogen (obtained from the pokeweed plant, and which also stimulates B

cells) (Oppenheim and Schechter, 1980; Tizard, 1986). The adequacy of all these mitogens for stimulation of ovine lymphocytes has been previously described (Burrells and Wells, 1977). Con A was the mitogen employed in this study.

Assays of lymphocyte transformation provide a simple, rapid and quantitative indication of cell-mediated immunity (Oppenheim *et al*, 1970; Catalona *et al*, 1975). The conventional assay for T-lymphocyte proliferation is based on incorporation of radioactive thymidine into the DNA of activated lymphocytes (Maluish and Strong, 1986). An alternative reliable method to assess T lymphocyte proliferation is the analysis of early activation markers, such as IL-2R, by flow cytometry (Prince and John, 1986). IL-2R expression is a prerequisite for DNA synthesis by activated lymphocytes and hence, the study of surface IL-2R expression on these cells will be a reliable indicator of proliferative responsiveness (Miyakawa *et al*, 1982).

5.3. EXPERIMENTAL DESIGN

Seven adult Texel ewes (age range 3-7 years), seropositive to MVV by the AGIDT (Winward *et al*, 1979), and 9 breed, sex and age-matched seronegative controls ewes were used (Chapter 2). Different animals from those employed in Chapters 3 and 4 were selected for this study. BALF and PB lymphocytes obtained from these animals were cultured for 3 days both in the presence and absence of Con A (experimental culture conditions are described in sections 2.12.4 and 2.12.5). The level of IL-2R expression by the cultured lymphocytes was then compared, both within groups (i.e. comparison of IL-2R expression in unstimulated and Con A-stimulated BALF and PB lymphocytes) and between groups (i.e. comparison of IL-2R expression by cells from MVV-infected and control animals).

5.4. MONOCLONAL ANTIBODIES AND REAGENTS

The same biotinylated mouse MAbs described in Chapter 4 were used in this study, namely SBU-T4 (anti CD4), SBU-T8 (anti CD8) [Maddox *et al*, 1985b] and CC-15 (anti- $\gamma\delta$ T cells) [Mackay *et al*, 1991].

The activation marker used on cultured lymphocytes was the bovine MAb IL-A111 (Naessens *et al*, 1992) against IL-2R- α .

Combinations of phycoerythrin labelled streptavidin (Amersham International, Amersham, U.K.) and rabbit antimouse IgG₁ FITC (The Binding Site, Birmingham, U.K.) were employed as secondary step reagents.

All the MAbs and reagents were used at previously determined optimal working dilutions (Table 4.2).

5.5. STATISTICAL ANALYSIS

As data were non normally distributed, BALF and PB sample comparisons were made using the Mann-Whitney non-parametric Test, with a significance level of 95%.

5.6. RESULTS

5.6.1 EFFECT OF Con A-STIMULATION ON CELL SIZE

BALF and PB lymphocytes from both groups of animals, following 3 days culture in the presence of Con A were of intermediate to large size and blastoid in morphology (Fig. 5.1). They showed an increase in size and complexity when compared with those unstimulated cells as measured by FSC and SSC (Fig. 5.2, Appendices I.5.1 and I.5.2).

Figure 5.1. Blastoid morphology of BALF lymphocytes from a control animal cultured *in vitro* for three days in the presence of Con A (5 μ g/ml). Most lymphoblasts were intermediate to large in size.

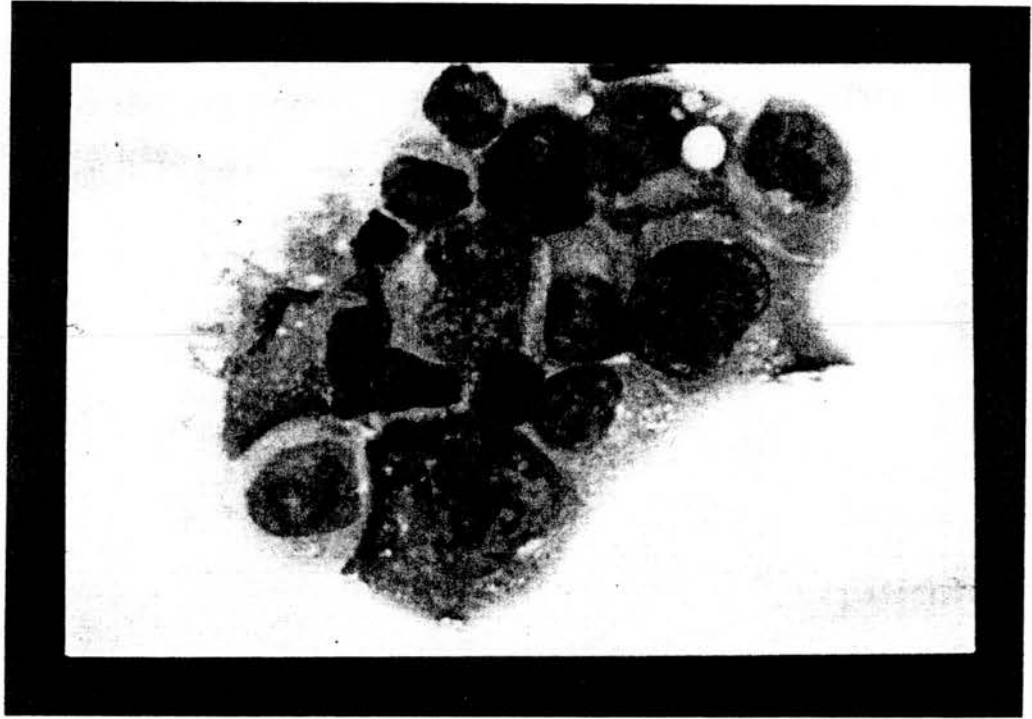
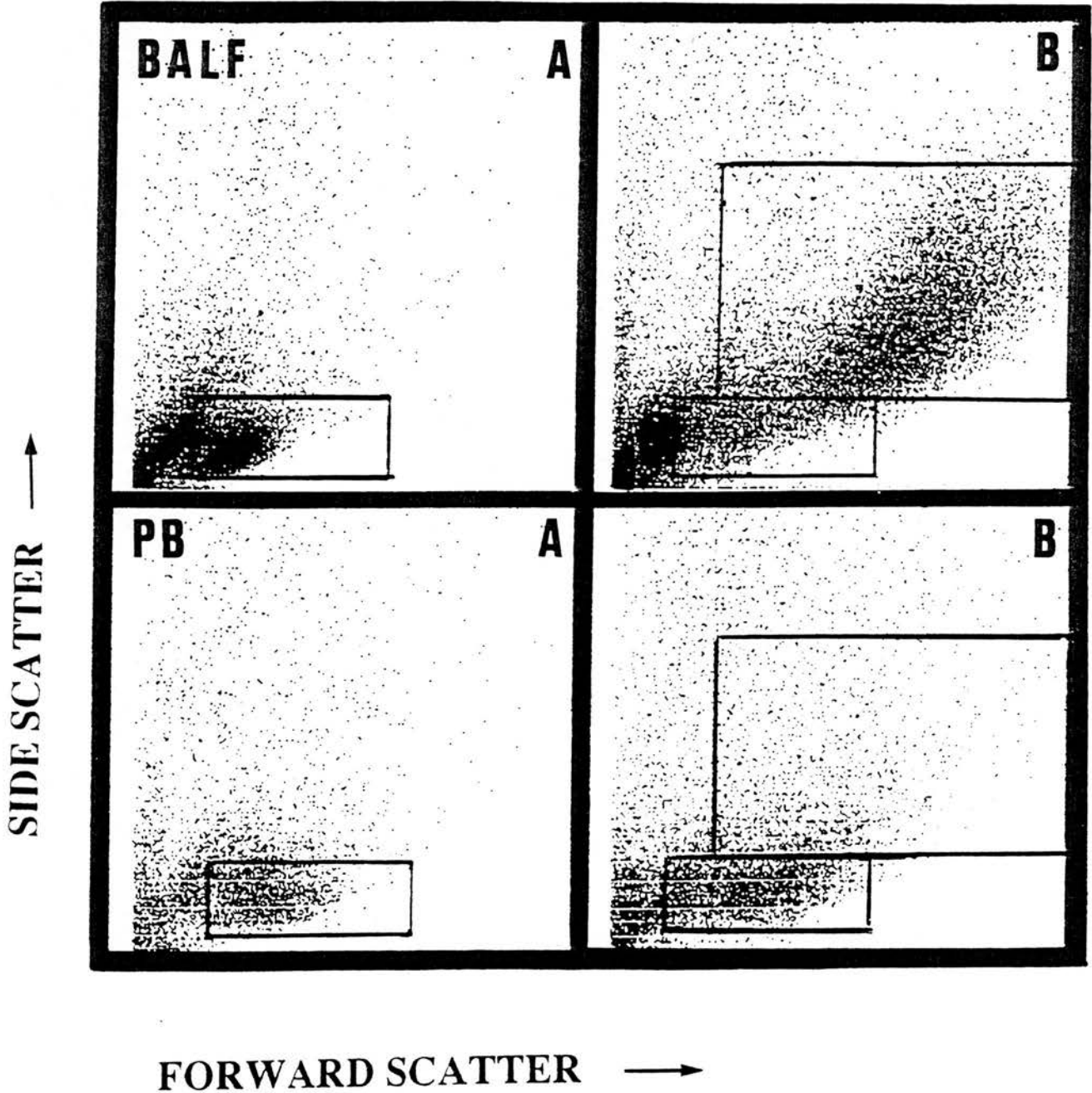


Figure 5.2. Effect of Con A-stimulation on cell size. Flow cytometry scatter profile of ovine BALF lymphocytes (top) and PB lymphocytes (bottom) after three days culture in the absence (A) and in the presence of Con A ($5\mu\text{g/ml}$)(B). Small lymphocytes are distinguished by their characteristic low forward scatter (FSC) and side scatter (SSC), lower boxes. Lymphoblasts are distinguished by their characteristic high FSC/SSC, upper boxes. BALF and PB lymphocytes cultured for three days in the presence of Con A (B) increased their size when compared with unstimulated lymphocytes (A), cultured for the same length of time.



Con A-stimulated BALF lymphocytes from MVV-infected animals had significantly increased FSC and SSC. BALF lymphocytes from control animals also had increased FSC and SSC parameters after Con A stimulation, but this increase was not significant (Table 5.1).

Table 5.1. Median of forward (FSC) and side (SSC) light scatter parameters of the gates established for unstimulated (No Con A) and Con A-stimulated BALF lymphocytes in control (n=9) and MVV-infected (n=7) animals. FSC values refer to cell size, while SSC values relate to cell complexity and internal structure. All cells were cultured *in vitro* for 3 days prior to analysis. Mann-Whitney non parametric rank test.

	<u>CONTROLS</u>	<u>MVV-INFECTED</u>
No Con A	FSC: 39,149 SSC: 6,57	FSC: 43,156 SSC: 8,57
Con A	FSC: 42,160 SSC: 7,71	FSC: 43,208* SSC: 8,78*

* Significantly different from unstimulated cells at $p < 0.05$.

Con A-stimulated PBL from both control and MVV-infected animals had significantly increased FSC and SSC parameters (Table 5.2).

Table 5.2. Median of forward (FSC) and side (SSC) light scatter parameters of the gates established for unstimulated (No Con A) and Con A-stimulated PBL in control (n=9) and MVV-infected (n=7) animals. FSC values refer to cell size, while SSC values relate to cell complexity and internal structure. All cells were cultured *in vitro* for 3 days prior to analysis. Mann-Whitney non parametric rank test.

	<u>CONTROLS</u>	<u>MVV-INFECTED</u>
No Con A	FSC: 47,173 SSC: 6,52	FSC: 40,184 SSC: 6,63
Con A	FSC: 50,226* SSC: 8*,95*	FSC: 45,223* SSC: 11*,123*

* Significantly different from unstimulated cells at $p < 0.05$.

5.6.2 COMPARISON OF WHOLE BALF CELLS vs MACROPHAGE-DEPLETED BALF CELLS

Macrophages, amid their multiple function can control both positively and negatively lymphocyte growth and differentiation (Unanue, 1981) and lymphocyte responses to antigens and mitogens (Lipscomb *et al*, 1982). Previous studies have shown that the proliferative response is a complex function depending on several factors such as numbers of macrophages and duration of culture (Liu *et al*, 1984).

To standardise our technique, IL-2R expression on cultured lymphocytes obtained from whole BALF was compared with that from lymphocytes recovered from BALF partially depleted of macrophages (as described in section 2.12.2). BALF lymphocytes from two controls and one MVV-infected sheep were employed for this study. After depletion of macrophages, mitogen stimulated BALF lymphocytes

expressed higher levels of IL-2R (Table 5.3). These results suggest that BALF macrophages interfere with the expression of IL-2R by BALF lymphocytes. Depletion of BALF macrophages was, therefore, routinely carried out.

Table 5.3. Comparison of IL-2R expression on BALF lymphocytes from whole BALF and from partially macrophage-depleted BALF in two control and one MVV-infected animal. Cells were cultured *in vitro* for 3 days prior to analysis. Results are expressed as percentages of positively stained cells within the total lymphocyte population.

		SHEEP NUMBER		
		C 92	C 98	MVV 28
MACROPHAGE DEPLETION	CON A STIMULATION	%IL-2R		
-	-	4.30	5.32	1.50
-	+	9.63	6.47	10.71
+	-	3.39	6.16	12.76
+	+	13.30	16.76	28.32

5.6.3 *IN VITRO* STIMULATION OF BALF LYMPHOCYTES

5.6.3.1 Within group effects of Con A stimulation

Controls: The expression of IL-2R was significantly increased on Con A-stimulated CD4⁺ ($p < 0.001$), CD8⁺ ($p < 0.001$) and $\gamma\delta$ T cells ($p < 0.05$) when compared with unstimulated cells (Table 5.4). The percentage of IL-2R expressing cells within the total lymphocyte population was not significantly increased after Con A stimulation (Table 5.4, Figs. 5.3 and 5.4).

MVV-infected animals: The expression of IL-2R was significantly increased on Con A-stimulated CD4⁺ and CD8⁺ T lymphocytes ($p < 0.05$) when compared with unstimulated cells (Table 5.5). The percentage of $\gamma\delta$ T cells expressing IL-2R was also increased after Con A stimulation, but this increase was not statistically significant. The percentage of IL-2R expression within the total lymphocyte population was significantly ($p < 0.05$) elevated after Con A stimulation (Table 5.5, Figs. 5.3 and 5.4).

5.6.3.2 Between group (MVV vs controls) effects of Con A stimulation

5.6.3.2.1 Total IL-2R expression

No significant differences were found in the overall percentage of BALF lymphocytes expressing IL-2R between control and MVV-infected animals, either in the absence of Con A or after Con A stimulation (Tables 5.6 and 5.7).

5.6.3.2.2 Expression of IL-2R by CD4⁺ T lymphocytes

The percentage of CD4⁺ T lymphocytes expressing IL-2R after Con-A stimulation was significantly ($p < 0.05$) lower in BALF from MVV-infected animals when compared to that of the controls (Table 5.7, Fig. 5.6).

5.6.3.2.3 Expression of IL-2R by CD8⁺ T lymphocytes

Prior to Con A stimulation, the percentage of CD8⁺ cells expressing IL-2R in BALF from MVV-infected animals was significantly lower ($p < 0.05$) than that from control animals (Table 5.6). After Con A stimulation, expression of IL-2R by CD8⁺ BALF T lymphocytes from MVV-infected animals was not significantly different from that of the controls (Table 5.7, Fig. 5.7).

5.6.3.2.4 Expression of IL-2R by $\gamma\delta$ T lymphocytes

As with CD8⁺ T lymphocytes, the percentage of unstimulated BALF $\gamma\delta$ T cells expressing IL-2R in MVV-infected animals was significantly lower ($p < 0.05$) than that of the controls (Table 5.6). Following Con A stimulation, expression of IL-2R by BALF $\gamma\delta$ T lymphocytes was not significantly different from that of control animals (Table 5.7).

5.6.3.2.5 Coexpression of CD4⁺ and CD8⁺ by Con A-stimulated BALF lymphocytes

Two-colour immunofluorescence labelling with the biotinylated anti-CD8 MAb, SBU-T8 and the unconjugated anti-CD4 MAb 17D.13 and flow cytometry revealed that while a very small proportion of resting T lymphocytes from BALF expressed CD4 and CD8 simultaneously, after 3 days culture with Con A, the proportion of double positive T cells increased significantly (Table 5.8).

Table 5.8. Coexpression of CD4⁺/CD8⁺ by unstimulated (No Con A) and Con A-stimulated BALF lymphocytes from control (n=9) and MVV-infected (n=7) animals. All cells were cultured *in vitro* for 3 days. Results expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses.

	<u>CONTROL</u>	<u>MVV-INFECTED</u>
UNSTIMULATED	4.3 (1.2-16.1)	1.3 (0.3-3.3)
Con A-STIMULATED	16.9 (7.8-25.6)*	27.3 (4.7-33.4)*

* Significantly different from unstimulated cells at $p < 0.05$ (Mann-Whitney non parametric rank test).

5.6.4 IN VITRO STIMULATION OF PBL

5.6.4.1 Within group effects of Con A stimulation

Controls: The expression of IL-2R was significantly increased ($p < 0.05$) on Con A-stimulated $CD4^+$, $CD8^+$ and $\gamma\delta$ T lymphocytes when compared with unstimulated PBL. The percentage expression of IL-2R by all lymphocytes was also significantly increased ($p < 0.05$) after Con A stimulation (Table 5.9, Figs. 5.3, 5.5- 5.7).

MVV-infected animals: The expression of IL-2R was significantly increased on Con A-stimulated $CD4^+$ ($p < 0.05$), $CD8^+$ ($p < 0.001$) and $\gamma\delta$ T lymphocytes ($p < 0.05$) when compared with unstimulated cells. The percentage of all lymphocytes expressing IL-2R was also significantly ($p < 0.05$) elevated after Con A stimulation (Table 5.10, Figs. 5.3, 5.5- 5.7).

5.6.4.2 Between group (MVV vs controls) effects of Con A stimulation

5.6.4.2.1 Total IL-2R expression

The percent expression of IL-2R by PB lymphocytes from control sheep was not significantly different from that of MVV-infected sheep after culture in the absence, or presence, of Con A stimulation (Tables 5.11 and 5.12).

5.6.4.2.2 Expression of IL-2R by $CD4^+$ T lymphocytes

No significant difference in the percentage of $CD4^+$ lymphocytes expressing IL-2R was observed between cultured PBL from control and MVV-infected animals, after culture in the absence, or presence, of Con A stimulation (Tables 5.11 and 5.12, Fig. 5.6).

5.6.4.2.3 Expression of IL-2R by $CD8^+$ T lymphocytes

A lower proportion ($p < 0.05$) of unstimulated cultured $CD8^+$ lymphocytes from MVV-infected animals expressed IL-2R than from controls. After Con A

stimulation the percentage of CD8⁺ lymphocytes bearing IL-2R was not significantly different from that of the controls (Tables 5.11 and 5.12, Fig. 5.7).

5.6.4.2.4 *Expression of IL-2R by $\gamma\delta$ T lymphocytes*

The percentage of cultured $\gamma\delta$ T cells bearing IL-2R antigen was significantly increased ($p < 0.05$) in unstimulated PBL from MVV-infected animals when compared with controls. After Con A stimulation IL-2R expression by $\gamma\delta$ T lymphocytes was similar in both control and MVV-infected animals (Tables 5.11 and 5.12).

Table 5.4. Expression of IL-2R by unstimulated and Con A-stimulated bronchoalveolar lavage fluid lymphocytes and different T cell subsets from control animals. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

	Lymphocyte Population	CD4 ⁺	CD8 ⁺	γδ ⁺
Non-stimulated n=9	7.6 (5.4-32.6)	16.7 (5.1-35.1)	7.6 (3.7-25.6)	42.4 (28.3-62.7)
Con A-stimulated n=9	21.0 (11.0-60.4)	81.7 ⁺ (66.0-96.3)	51.5 ⁺ (37.3-91.8)	56.7* (46.6-88.7)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

*Significantly different from unstimulated at P<0.05 (Mann-Whitney non parametric rank test)

+Significantly different from unstimulated at P<0.001 (Mann-Whitney non parametric rank test)

Table 5.5. Expression of IL-2R by unstimulated and Con A-stimulated bronchoalveolar lavage fluid lymphocytes and different T cell subsets from MVV-infected animals. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

Lymphocyte Population		CD4 ⁺	CD8 ⁺	γδ ⁺
Non-stimulated n=7	8.4 (1.1-22.0)	12.5 (1.3-21.6)	1.8 (0.1-3.1)	14.1 (0.9-55.3)
Con A-stimulated n=7	20.1* (10.7-34.3)	50.0* (16.6-70.3)	37.3* (7.5-54.3)	48.7 (5.8-87.2)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

*Significantly different from unstimulated at P<0.05 (Mann-Whitney non parametric rank test)

Table 5.6 Expression of IL-2R by unstimulated bronchoalveolar lavage fluid lymphocytes and different T cell subsets from control and MVV-infected sheep. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

	Total %IL-2R	CD4/IL-2R	CD8/IL-2R	$\gamma\delta$ /IL-2R
CONTROL n=9	7.6 (5.4-32.6)	16.7 (5.1-35.1)	7.6 (3.7-25.6)	42.4 (28.3-62.7)
MAEDI n=7	8.4 (1.1-22.0)	12.5 (1.3-21.6)	1.8* (0.1-3.1)	14.1* (0.9-55.3)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

*Significantly different from controls at $P < 0.05$ (Mann-Whitney non parametric rank test)

Table 5.7. Expression of IL-2R by Con A-stimulated bronchoalveolar lavage fluid lymphocytes and different T cell subsets from control and MVV-infected sheep. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

	Total	% IL-2R	CD4/IL-2R	CD8/IL-2R	$\gamma\delta$ /IL-2R
CONTROL n=9	21.0 (11.0-60.4)	81.7 (66.0-96.3)	51.5 (37.3-91.8)	56.7 (46.6-88.7)	
MAEDI n=7	20.1 (10.7-34.3)	50.0* (16.6-70.3)	37.3 (7.5-54.3)	48.7 (5.8-87.2)	

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

*Significantly different from controls at $P<0.05$ (Mann-Whitney non parametric rank test)

Table 5.9. Expression of IL-2R by unstimulated and Con A-stimulated peripheral blood lymphocytes and different T cell subsets from control animals. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

Lymphocyte Population		CD4 ⁺	CD8 ⁺	γδ ⁺
Non-stimulated n=7	7.5 (2.0-11.9)	18.1 (4.5-28.9)	6.7 (1.3-26.9)	6.7 (2.0-26.5)
Con A-stimulated n=7	35.3* (9.5-52.5)	69.1* (23.4-95.9)	29.1* (11.5-69.9)	57.3* (8.4-98.4)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

* Significantly different from unstimulated at P<0.05 (Mann-Whitney non parametric rank test)

Table 5.10. Expression of IL-2R by unstimulated and Con A-stimulated peripheral blood lymphocytes and different T cell subsets from MVV-infected animals. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

	Lymphocyte Population	CD4 ⁺	CD8 ⁺	γδ ⁺
Non-stimulated n=8	5.7 (2.6-17.8)	22.6 (16.5-64.0)	1.3 (0.6-8.7)	18.1 (7.6-31.5)
Con A-stimulated n=8	28.1* (2.1-38.9)	73.4* (22.8-92.3)	48.9+ (10.1-66.1)	60.1* (21.6-75.9)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

*Significantly different from unstimulated at P<0.05 (Mann-Whitney non parametric rank test)

+Significantly different from unstimulated at P<0.001 (Mann-Whitney non parametric rank test)

Table 5.11. Expression of IL-2R by unstimulated peripheral blood lymphocytes and different T cell subsets from control and MVV-infected sheep. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

	Total	%IL-2R	CD4/IL-2R	CD8/IL-2R	$\gamma\delta$ /IL-2R
CONTROL n=7	7.5 (2.0-11.9)	18.1 (4.5-28.9)	6.7 (1.3-26.9)	6.7 (2.0-26.5)	
MAEDI n=8	5.7 (2.6-17.8)	22.6 (16.5-64.0)	1.3* (0.6-8.7)	18.1* (7.6-31.5)	

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

* Significantly different from controls at $P < 0.05$ (Mann-Whitney non parametric rank test)

Table 5.12. Expression of IL-2R by Con A-stimulated peripheral blood lymphocytes and different T cell subsets from control and MVV-infected sheep. Lymphocytes were cultured *in vitro* for 72 h prior to analysis.

	Total % IL-2R	CD4/IL-2R	CD8/IL-2R	γδ/IL-2R
CONTROL n=7	35.3 (9.5-52.5)	69.1 (23.4-95.9)	29.2 (11.5-69.9)	57.3 (8.4-98.4)
MAEDI n=8	28.1 (2.1-38.9)	73.4 (22.8-92.3)	48.9 (10.1-66.1)	60.1 (21.6-75.9)

Results are expressed as median of percentages of double-positively labelled cells in lymphocyte gate with ranges in parentheses

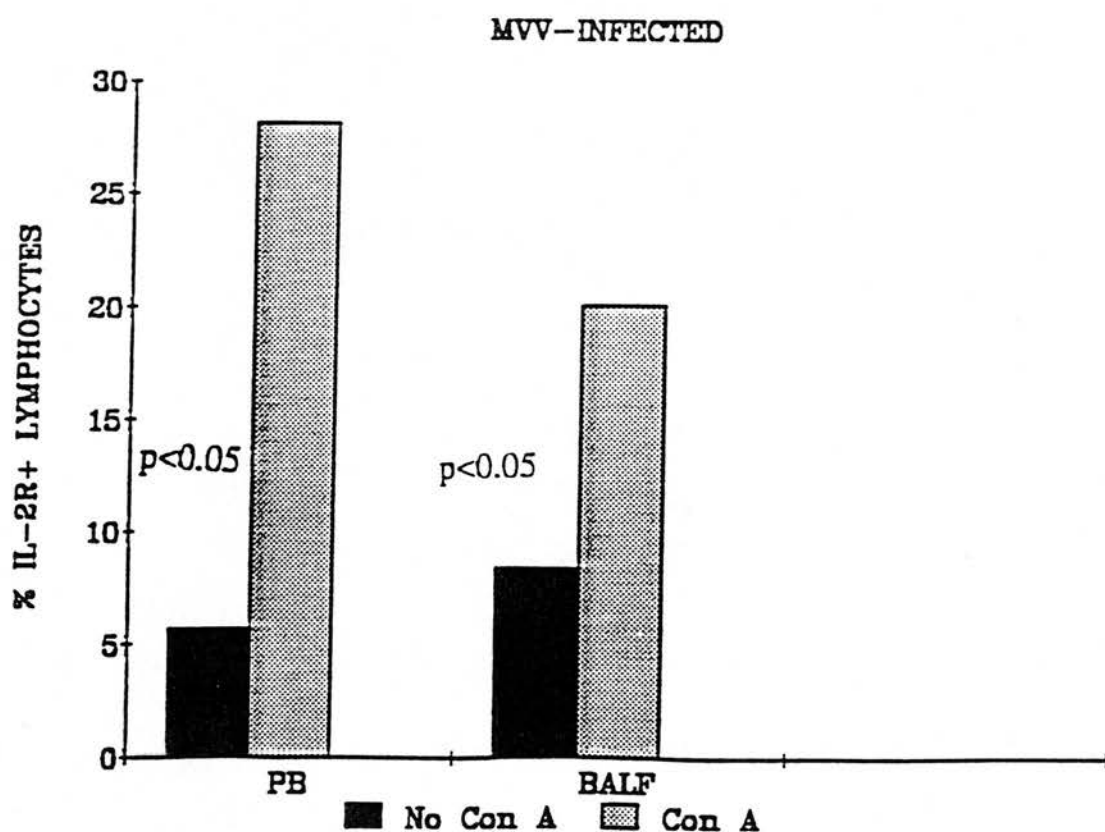
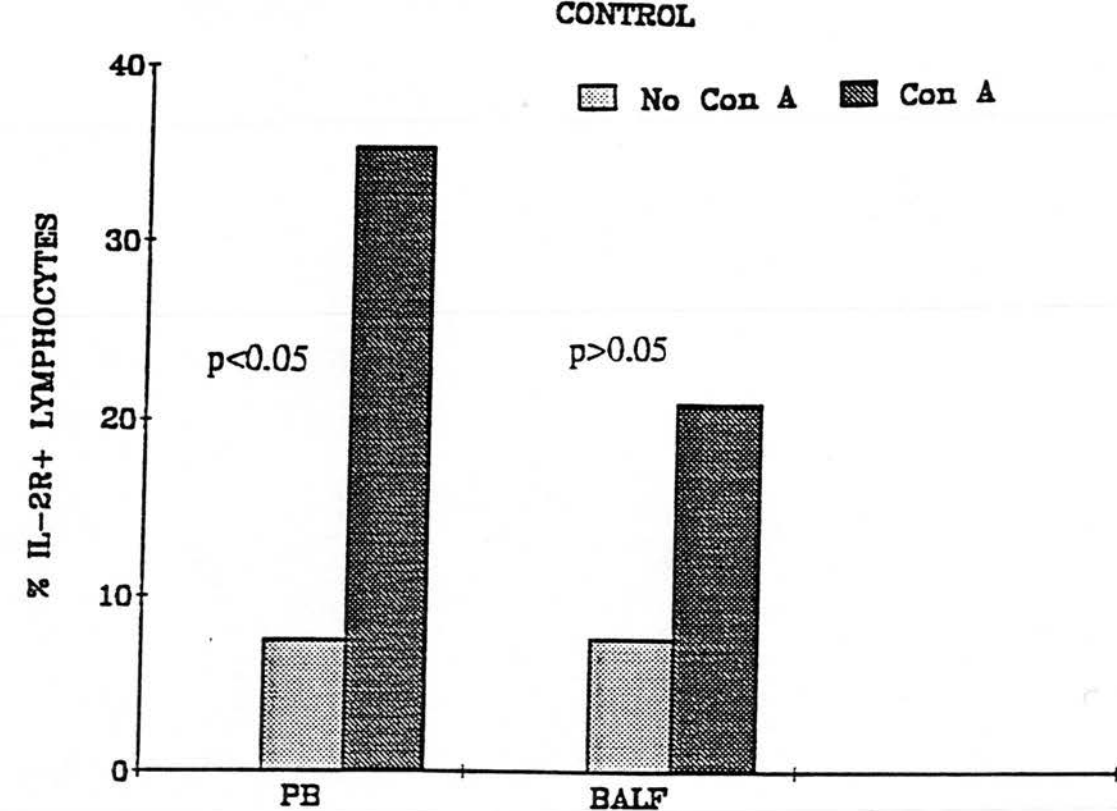


Figure 5.3. IL-2R expression of PB and BALF lymphocytes from control (n=9) and MVV-infected sheep (n=7) after TCR stimulation *in vitro* with Con A. Data are shown as median of percentages of IL-2R⁺ lymphocytes after 72h in culture with or without Con A stimulation. Mann-Whitney non parametric rank test.

Figure 5.4. Fluorescence histograms of surface expression of IL-2R on unstimulated (2) and Con A-stimulated (5 μ g/ml) (3) BALF lymphocytes from a control (n^o BT 70) and a MVV-infected (n^o 68) sheep. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and linear cell frequency is given on the ordinate. Normal mouse serum (NMS) was used as the negative control (1).

After culture for three days, a higher increase in the percentage of IL-2R was observed in those BALF lymphocytes stimulated with Con A

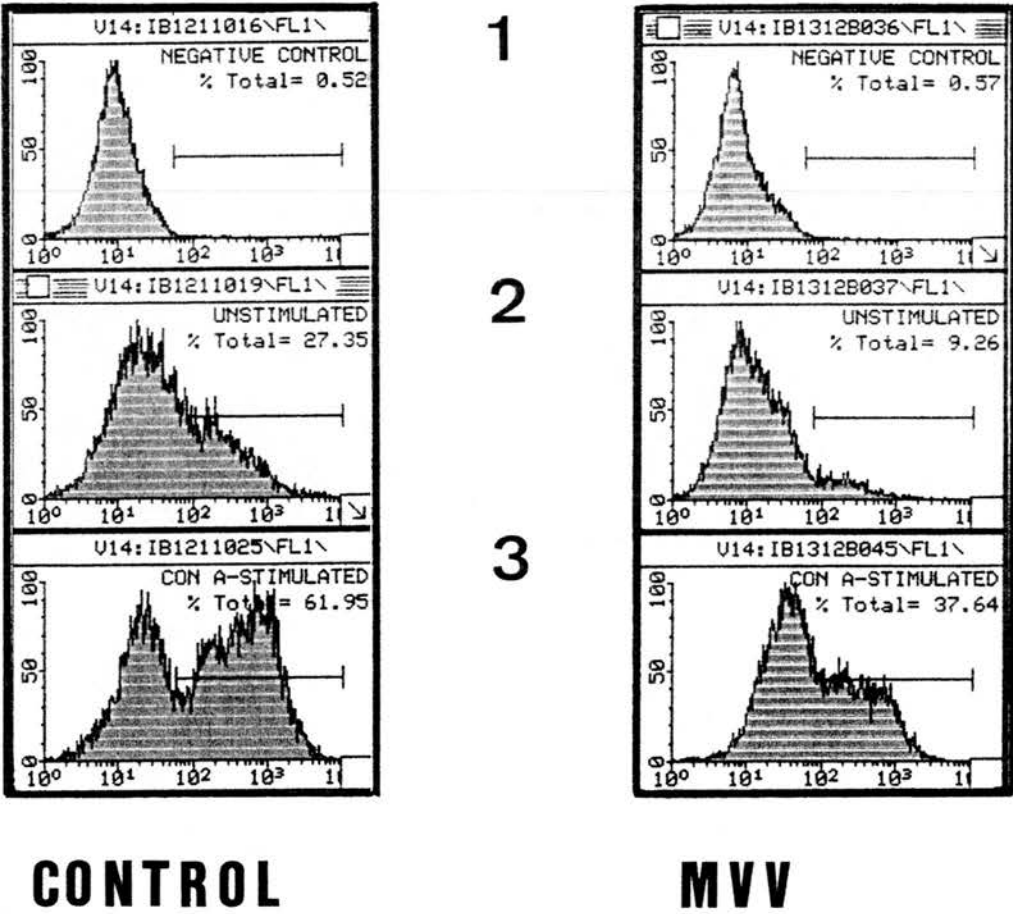
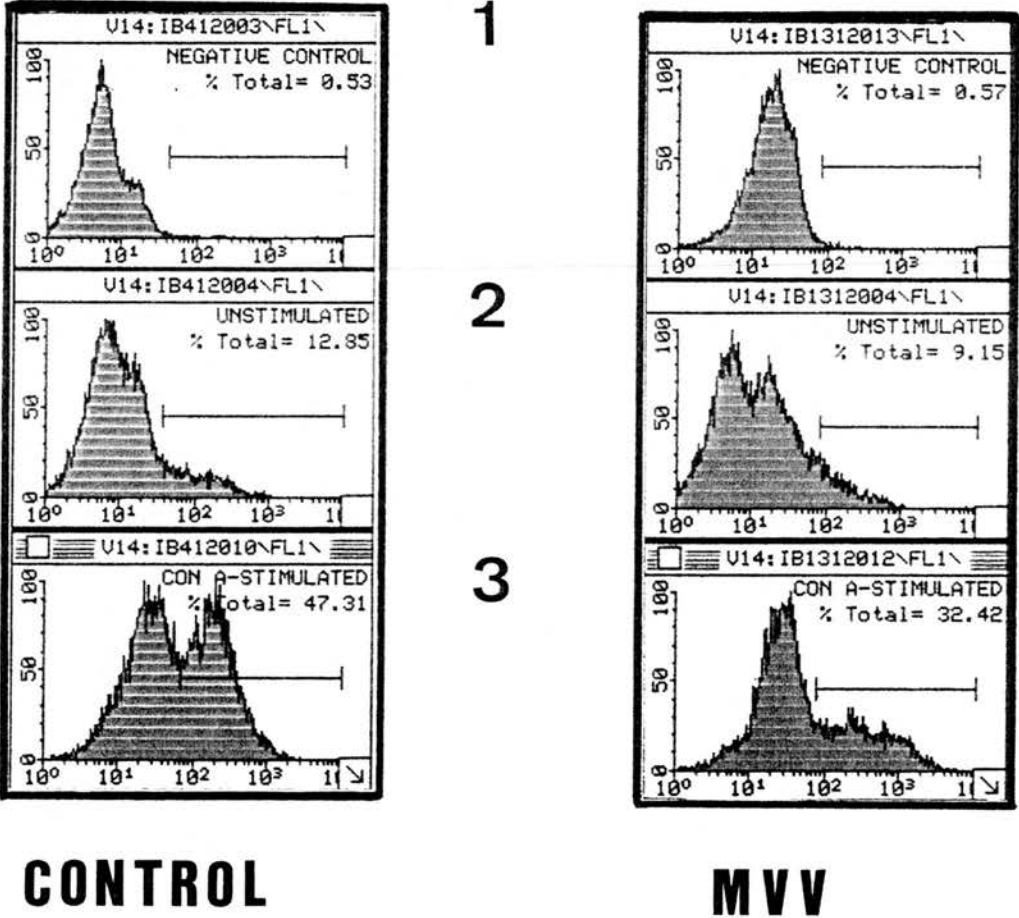


Figure 5.5. Fluorescence histograms of surface expression of IL-2R on unstimulated (2) and Con A-stimulated (5 μ g/ml) (3) PBL from a control (n^o MS 28) and a MVV-infected (n^o 36) sheep. Logarithmic fluorescence intensity is plotted on the abscissa in arbitrary units and linear cell frequency is given on the ordinate. Normal mouse serum (NMS) was used as the negative control (1).

After culture for three days, a higher increase in the percentage of IL-2R was observed in those BALF lymphocytes stimulated with Con A



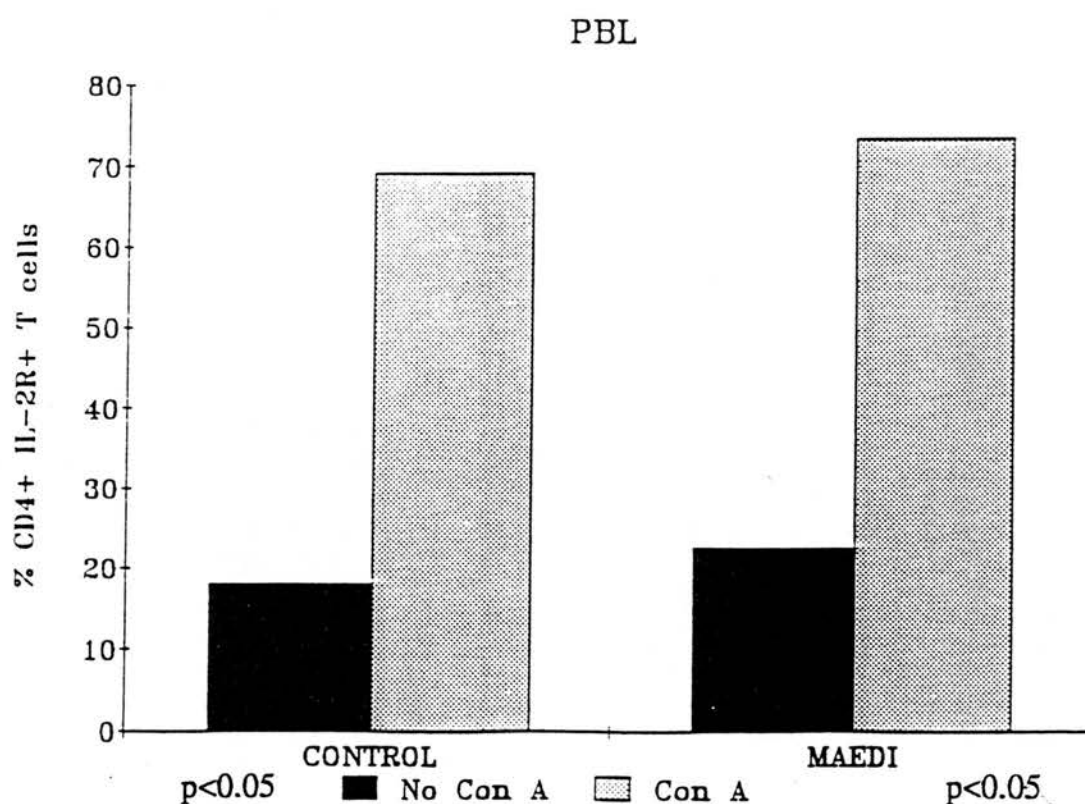
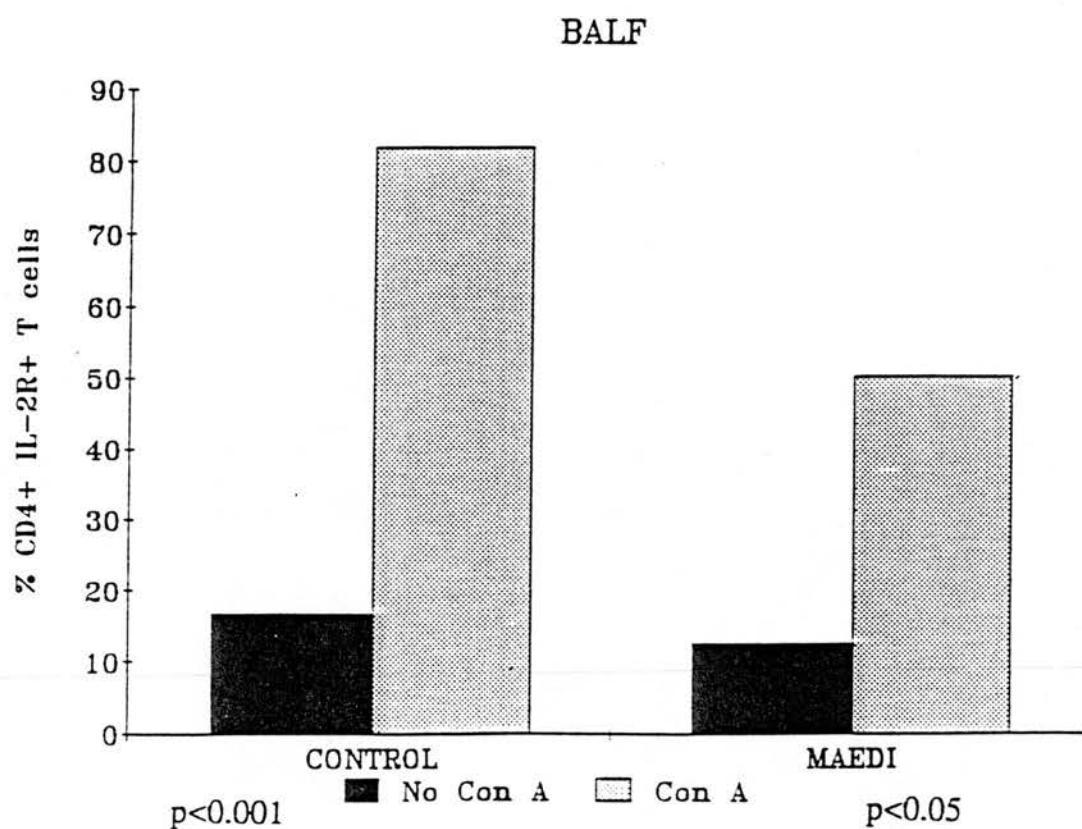


Figure 5.6. Con A induced expression of IL-2R on CD4⁺ BALF and PB lymphocytes from control (n=9) and MVV-infected (n=7) animals. Data are presented as median of the percentages of IL-2R⁺ CD4⁺ T cells after 72 h of culture unstimulated (No Con A) or stimulated with 5µg/ml of Con A. Mann-Whitney non parametric rank test.

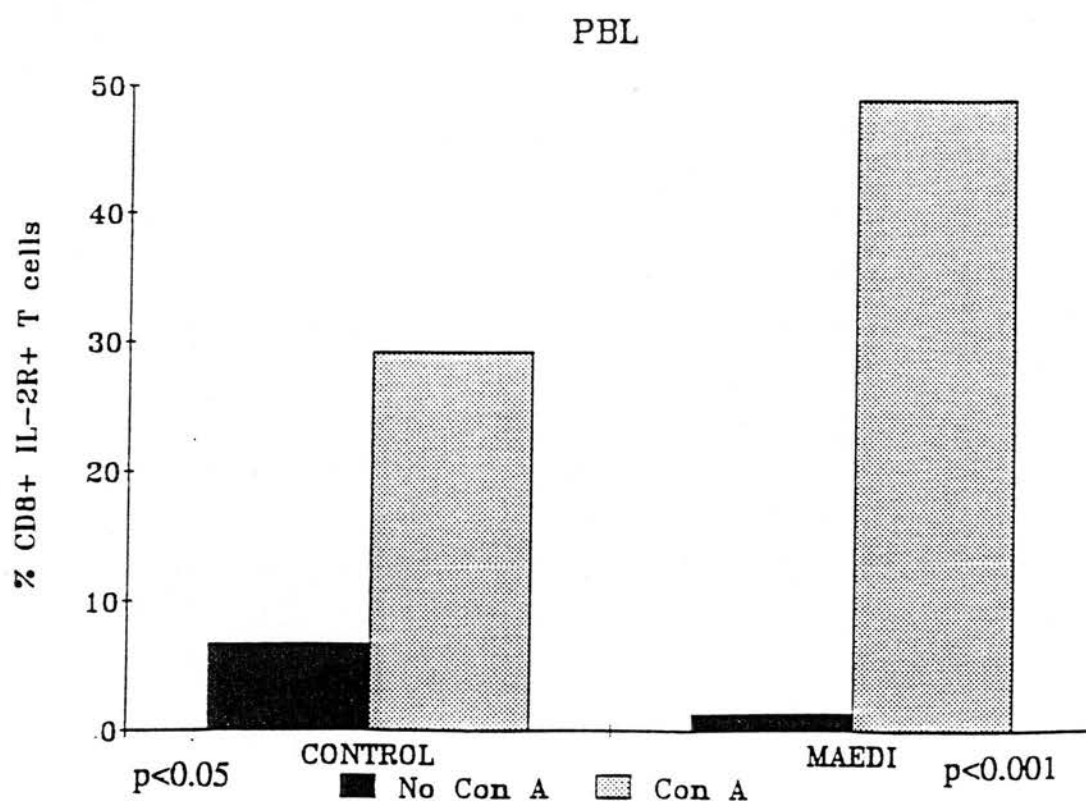
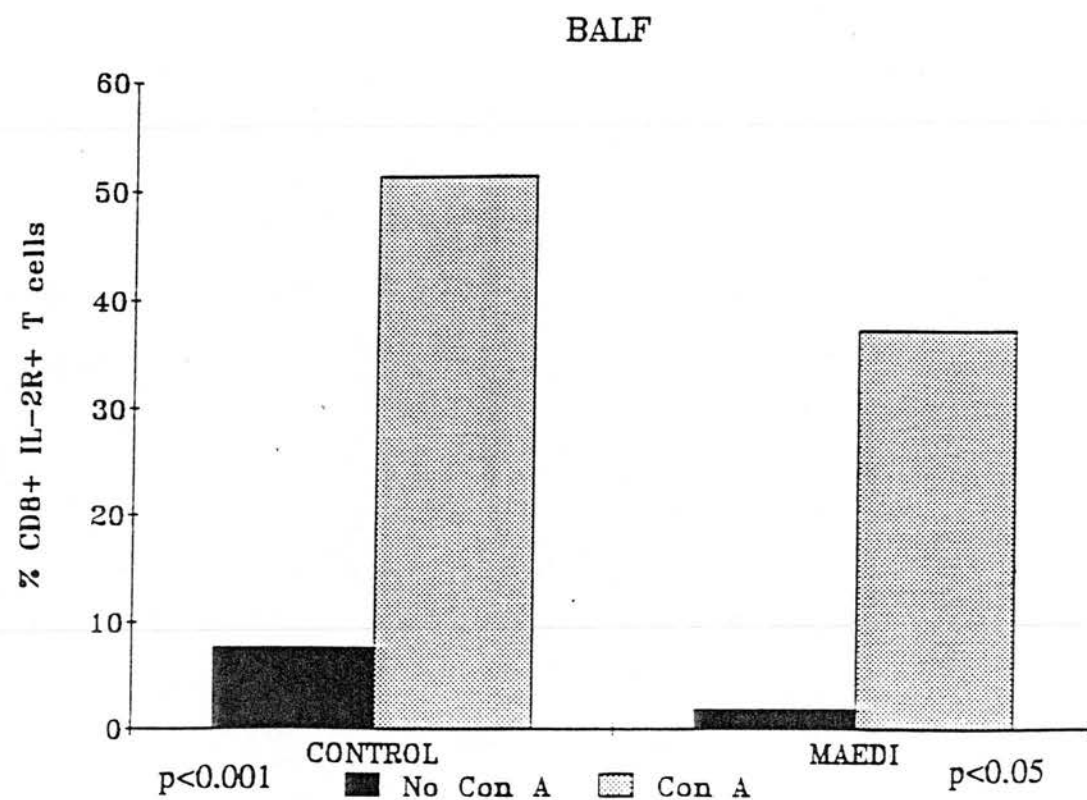


Figure 5.7. Con A induced expression of IL-2R on CD8⁺ BALF and PB lymphocytes from control (n=9) and MVV-infected (n=7) animals. Data are presented as median of the percentages of IL-2R⁺ CD8⁺ T cells after 72 h of culture unstimulated (No Con A) or stimulated with 5µg/ml of Con A. Mann-Whitney non parametric rank test.

5.7. DISCUSSION

Our previous studies (Chapter 4) on the stage of activation of BALF and PB T lymphocytes from MVV-infected animals suggested an impaired BALF T lymphocyte activation during the course of MVV infection. The aim of this experiment was to determine whether the anomalous pattern of lymphocyte activation observed during the course of the natural disease was maintained under *in vitro* conditions. The expression of IL-2R (an early marker of lymphocyte activation) by *in vitro* cultured BALF and PB lymphocytes in response to exogenous mitogen stimulation was studied.

In this work, and for both groups of animals, the percentage of all unstimulated BALF and PB T cells which expressed IL-2R differed from values reported in Chapter 4 (Tables 4.4-4.8). In Chapter 4, however, IL-2R expression by BALF and PB lymphocytes was studied immediately after isolation of the cells, while in the present work lymphocytes were cultured for 72 h *in vitro* prior to analysis. The *in vitro* culture procedure could explain this difference.

Cell proliferation in response to various mitogens is commonly employed to investigate the functional integrity of lymphocytes (Rola-Pleszcynski *et al*, 1981). Characterisation of the blastogenic responses of ovine lymphocyte suspensions to certain mitogens has already been performed (Burrells and Wells, 1977).

Responses of pulmonary lymphocytes from humans (Daniele *et al*, 1975), monkeys (Kazmierowski *et al*, 1976) and sheep (Rola-Pleszcynski *et al*, 1981) to mitogens have been reported. IL-2R expression by activated lymphocytes after mitogen stimulation has also been described (Kaczmarek, 1986).

The poor response of BALF lymphocytes to mitogens previously described by some authors (Burrells, 1985b) was not observed in this study, as BALF cultured lymphocytes from both control and MVV-infected animals responded to Con A stimulation with proliferation and increase in size. After partial depletion of BALF macrophages, the expression of IL-2R by Con A-stimulated BALF lymphocytes from control and MVV-infected animals was increased, suggesting that BALF macrophages have an inhibitory effect on BALF lymphocyte function (Liu *et al*, 1984). The poor response of ovine BALF lymphocytes in Burrells' study (1985b) cannot be explained by the inhibitory effect of BALF macrophages, as BALF phagocytic cells were removed by carbonyl iron treatment prior to BALF cell culture.

Considerable heterogeneity was found in the stimulus-response relationship exhibited by individual BALF and PB T cells from both control and MVV-infected animals. This individual variation to mitogens was greater in the case of PB T cells as was previously described (Burrells, 1985b). Although the biochemical basis of this heterogeneity is currently unknown, it has been suggested that it could be due to the individual differences of T cells in their requirements for activation (Geppert *et al*, 1990).

Following *in vitro* Con A stimulation, ovine BALF and PB T lymphocytes from both control and MVV-infected animals increased their expression of IL-2R, demonstrating that ovine T cells are immunocompetent, responding to activation signals and expressing IL-2R in a manner similar to T lymphocytes from other species (Rideout *et al*, 1992) and arguing against an acquired or constitutive dysfunction of the T cell population. The fact that those cells, which *in vivo* showed very low detectable levels of IL-2R, increased these levels after mitogen stimulation with Con

A, indicates their normal response to the stimulation of the TCR/T3 complex by the mitogen. This supports the interpretation that they had already undergone a prior activation step, and were prepared to undergo blast transformation. These preactivated T cells could have expressed IL-2R levels below the threshold necessary for detecting immunofluorescence staining by flow cytometry (Burmester *et al*, 1984).

However, IL-2R expression by cultured unstimulated BALF T lymphocytes from controls differed from that of cells from MVV-infected animals. Cultured unstimulated BALF T lymphocytes from control animals expressed higher levels of IL-2R than cultured unstimulated BALF T lymphocytes from MVV-infected animals, those differences being significant in the case of the CD8⁺ and $\gamma\delta$ T cell subsets. Differences in IL-2R expression on BALF T lymphocytes from both groups of animals might have occurred prior to any process of stimulation.

In both control and MVV-infected animals, cultured Con A-stimulated BALF lymphocytes in all T cell subsets had increased IL-2R expression when compared with their unstimulated controls. However, IL-2R expression by Con A-stimulated CD4⁺ T cells from MVV-infected animals remained significantly lower than that of Con A-stimulated CD4⁺ T cells from controls.

BALF CD4⁺ T lymphocytes from MVV-infected animals, despite undergoing mitogen stimulation did not attain the same level of IL-2R expression as control BALF CD4⁺ T lymphocytes. The capacity of BALF CD4⁺ T lymphocytes from MVV-infected animals to respond could be diminished by multiple mechanisms, such as by soluble factors present in the PELF. Although it has been reported that suppression of immunity and severe lymphoid depletion are not prominent features of ovine lentiviral infection of sheep (Pearson *et al*, 1989), this experiment shows that

impaired cellular immunity may be affecting some BALF T lymphocyte subsets from MVV-infected animals. Not only did BALF T lymphocytes from MVV-infected animals fail to respond normally to activation (as suggested in Chapter 4), but IL-2R expression was also reduced in unstimulated BALF CD8⁺ and $\gamma\delta$ T lymphocytes *in vitro*.

These results in BALF lymphocytes are concordant with those from Ellis and DeMartini (1985b), who found decreased responses to Con A stimulation in PB and pulmonary lymph node lymphocytes from adult sheep with naturally occurring ovine lentiviral infection.

BALF T cells from MVV-infected animals may follow a similar pattern to AIDS infected human T cells which, during the course of *in vivo* infection, do not express the Tac antigen, although it is possible to detect this receptor after *in vitro* mitogen stimulation (Lane and Fauci, 1985). Decreased lymphoproliferative responses to mitogens and soluble antigens in patients with AIDS and related disorders have been described (Schroff *et al*, 1983; Lane and Fauci, 1985; Prince and John, 1986). Cats infected with FIV also manifest an inability to respond to mitogens characterised by a defect in IL-2 production in secondary lymphoid organs (Lawrence *et al*, 1992).

The decreased response to activation observed in BALF lymphocytes from MVV-infected animals could play an important role in the pathogenesis of MVV infection. Hence, the poor response to mitogens could be related to defective IL-2 production as reported in AIDS studies (Munn *et al*, 1984; Prince *et al*, 1984; Ebert *et al*, 1985; Gupta, 1986). This would cause an impaired lung immune defence during MVV infection.

Regarding the coexpression of CD4 and CD8 by Con A-stimulated BALF lymphocytes, our findings are concordant with previous studies in normal humans where small percentages of BALF cells were positive for both CD4 and CD8 (Davidson *et al*, 1985; Becker *et al*, 1990). Concurrent expression of CD4 and CD8 is the rule in stage II maturing thymocytes, which further differentiate in the thymus, segregating into reciprocal CD4⁺ and CD8⁺ subsets (Davidson *et al*, 1985). The functional role of these subsets is unclear, although it seems to be complex and heterogeneous, with cytotoxic cells present in both subsets and the CD4⁺ subset containing both suppressor/inducer and helper functions (Romain and Schlossman, 1984).

It has been reported that, after *in vitro* culture, cells tend to acquire unusual properties, such as the expression of both CD8 and CD4 (Tite and Janeway, 1984; Oliveira and Mitchison, 1989). Some of these cells might represent T helper cells that have lost their helper function and are now acting as cytotoxic cells.

Individual PBL from both control and MVV-infected animals, as previously mentioned, showed a greater heterogeneity in the response to Con A than BALF lymphocytes from the same animals. In concordance with what happened in BALF, Con A stimulation increased the expression of IL-2R on all the T cell subsets in both groups of animals. However, when comparing IL-2R expression between PBL from control and MVV-infected animals, results were distinct from those obtained for BALF. Hence, while lower percentages of unstimulated CD4⁺ and $\gamma\delta$ ⁺ T cells from control animals expressed IL-2R, higher percentages of unstimulated CD8⁺ T cells from these animals expressed IL-2R. Following Con A-stimulation, all PB T cell subsets from MVV-infected animals expressed higher IL-2R than those of controls. Results obtained for PBL were hence, erratic and difficult to interpret. These results differed from previous findings in which no differences between the level of response

of PBL to mitogens between control and lentivirus infected sheep were observed (Griffin *et al*, 1978; Sihvonen, 1981; Larsen *et al*, 1982b; Narayan *et al*, 1988).

CHAPTER 6: EXPERIMENTAL MVV INFECTION: EARLY CELLULAR IMMUNE RESPONSE

6.1. SUMMARY

A model of experimental infection with a lytic British isolate of MVV, designated EV1, was developed. Twelve Texel sheep were allocated to three groups and inoculated by the respiratory route with different inocula. Six of the animals received $10^{7.2}$ TCID₅₀ of MVV EV1 strain. Two sheep were challenged with heat inactivated MVV EV1 strain. An additional group of four sheep was sham-inoculated with identically prepared virus-free buffer solution, animals in this group serving as controls. Experimental infection was followed for 16 weeks post-inoculation (PI). Prior to inoculation, routine haematology, BAL and flow cytometric analyses of BALF lymphocytes were performed in all the animals to provide baseline parameters. Dual and single colour flow cytometric analyses of BALF lymphocytes and differential BALF cell counts were performed. Transforming growth factor- β (TGF- β) levels, in both BALF supernatant and plasma samples, were measured. Concentrations of pulmonary epithelial lining fluid (PELF) TGF- β were calculated.

Precipitating antibodies to MVV developed in the MVV-inoculated animals during the first 4 weeks PI. Changes in the cellular composition of BALF were observed in the three groups of animals during the first 2 weeks PI and persisted throughout all the sampling period. All groups showed increased percentages of BALF lymphocytes and decreased percentages of BALF macrophages, these changes being significant ($p < 0.05$) in the MVV-inoculated group when compared with the other two groups of animals 4 weeks PI. Phenotypic changes in BALF T lymphocytes from MVV-inoculated animals, compared with the other two groups, were characterised by significantly decreased ($p < 0.05$) percentages of CD4⁺, CD5⁺ and $\gamma\delta$ T lymphocytes, significantly increased ($p < 0.05$) percentages of CD8⁺ lymphocytes

and significant inversion ($p < 0.05$) of the $CD4^+/CD8^+$ ratio. All phenotypic changes in BALF T cell subsets occurred between 2 and 6 weeks PI, and a trend to return to baseline levels was evident at the end of the sampling period. Upregulated expression of MHC Class II and IL-2R molecules was observed on BALF T lymphocytes from the three groups of animals following inoculation.

TGF- β concentrations in PELF were approximately 165-fold higher than in plasma. No significant differences in the concentrations of plasma or PELF TGF- β , either within or among groups, were observed.

These findings indicate that an early, short term cellular immune response occurs during experimental MVV-infection.

6.2. INTRODUCTION

In spite of extensive work on the virology, serology and pathology of MVV (Gudnadottir, 1974; Georgsson *et al*, 1976; Thormar, 1976) many aspects of the early immune responses during MVV infection remain unexplained. The study of the acute events associated with experimental MVV infection of the lungs, is of considerable importance for understanding the subsequent course of the disease.

In this work, the development of an experimental pulmonary MVV infection in a group of sheep, and the repeated monitoring of the local responses by BAL, allowed the study of the early pulmonary immune response during ovine lentiviral infection.

The first successful experimental transmission of MVV was achieved by Gudnadottir and Palsson (1967). Lambs were inoculated with a suspension of MVV directly into the lungs, reproducing the pathology and clinical signs of visna. Since then, experimental infection of sheep with MVV has become a normal practice for the investigation of different aspects of the disease (Dawson *et al*, 1985; Geballe *et al*, 1985; Kennedy *et al*, 1985).

Some work on the early (Sihvonen, 1981; Larsen *et al*, 1982a; Cordier *et al*, 1992) and late (Sihvonen, 1981; Larsen *et al*, 1982b) cellular immune responses in sheep experimentally inoculated with MVV has already been performed. The present work, however, used different inoculation, sampling and investigation techniques from those from Sihvonen (1981) and from Larsen and others (1982a), and also different inoculation and sampling techniques from those of Cordier and others (1992).

Main differences between the present MVV-experimental infection and previous ones.

a) Inoculation and sampling techniques

With respect to the inoculation and sampling techniques, Sihvonen (1981) inoculated sheep with MVV, either intracerebrally or by respiratory route (either by direct injection of the viral suspension into the lungs or intranasally), while Larsen and others (1982a/b) used intraperitoneal, intramuscular, subcutaneous or intratracheal inoculations, and Cordier and others (1992) employed transtracheal inoculation.

In this study, fibreoptic endoscopy and BAL were used to inoculate the animals and to collect BALF samples. This technique enabled repetitive BAL sampling from the lung lobe into which the inoculum was delivered.

b) Investigation techniques and objectives pursued

Sihvonen (1981) and Larsen and others (1982a) employed a lymphocyte stimulation test and different serological assays for the study of the early humoral immune response. Larsen and others (1992a) also studied the responses of BCG-sensitised lymphocytes and lymphocyte responses to mitogens. Cordier and others

(1992) used flow cytometry and indirect immunohistochemistry to identify the lymphocyte subpopulations in BALF and lung tissue, respectively.

The present study used flow cytometry to monitor changes in phenotypes and in the state of activation of BALF T cell subsets. The main advantage of this study with respect to that of Cordier and others was the possibility of regular sampling, while Cordier and others collected BALF samples only once at slaughter. Another difference was the age of the animals, with adults being used in the present experiment and lambs in Cordier's study.

Additionally, the present study employed a bioassay for transforming growth factor β (TGF- β) to quantify pulmonary epithelial lining fluid (PELF) and plasma levels of TGF- β . The reason for analysing this particular cytokine was that due to the multifunctional role of TGF- β (both as an immunosuppressive and as a potent proinflammatory molecule) it could account for many of the pathological features observed during MVV-infection. Its chemotactic activity for both neutrophils (Brandes *et al*, 1991; Reibman *et al*, 1991) and T lymphocytes (Adams *et al*, 1991), could explain the accumulation of both types of cells in BALF (Chapter 3) and in the pulmonary interstitium (Watt *et al*, 1992b). The enhanced expression of LFA-1, on BALF lymphocytes (Chapter 3), could be mediated by the participation of TGF- β in cell-cell and cell-matrix interactions (Ignatz *et al*, 1989). The lack of IL-2R expression by activated BALF T lymphocytes (Chapter 4), could be a consequence of the ability of TGF- β to block IL-2 mediated induction of IL-2 receptors (Kehrl *et al*, 1986a; Ahuja *et al*, 1993). The role of TGF- β as an inhibitor of lymphocyte function (Wakefield *et al*, 1988) could explain the diminished activation of BALF lymphocytes from MVV-infected animals in response to mitogen stimulation (Chapter 5). Finally, TGF- β could account for the increased spontaneous release of fibronectin by alveolar

macrophages (Ignatz and Massague, 1986) described in MVV-infected sheep by Cordier and others (1990).

In summary, the present work is a dynamic study of the changes the ovine lower respiratory tract undergoes during experimental MVV-infection. Not only the appearance of changes, but also their timing are investigated. Neither the study of the progressive phenotypic changes in BALF T cell subsets, nor the quantification of TGF- β in ovine PELF had been described before.

6.2.1 CYTOKINES OF THE LUNG: TRANSFORMING GROWTH FACTOR (TGF- β)

Cytokines are secretory molecules released by cells that signal the replication and phenotypic changes of nearby cells. They are intimately involved in normal lung homeostasis (Kelley, 1990). Any anatomic or physiologic perturbation of the normally stable lung will lead immediately to a cascade of cell-to-cell signaling events mediated by the release of cytokines within the lung.

Those cytokines receiving the most attention to date in relation to pulmonary diseases include platelet derived growth factor, IL-1, TGF- β , tumor necrosis factor α (TNF- α), insulin-like growth factor I and IL-6.

TGF- β s are a family of multifunctional growth-modulating protein cytokines with capacity to modulate a wide range of cellular behaviours (Kelley, 1993). TGF- β is a dimeric protein of approximately 25 kDa. The two 12.5 kDa subunits are either identical or closely related polypeptides, depending on the cell source. TGF- β exists as three distinct homodimers, TGF- β_1 , TGF- β_2 and TGF- β_3 (Pelton *et al*, 1990). Mature TGF- β_3 has approximately 80% sequence homology with TGF- β_1 and TGF- β_2 , while TGF- β_1 shares approximately 71% homology with TGF- β_2 (Danielpour, 1989).

TGF- β can be extracted from most tissues, platelets being the most concentrated source of this molecule (Assoian *et al*, 1983). T lymphocytes, macrophages, monocytes, neutrophils and fibroblasts also express TGF- β constitutively. Normal and neoplastic tissues (Roberts *et al*, 1983) and most cells in culture can also synthesise varying amounts of TGF- β . Receptors for this cytokine have also been found in almost all known cell lines (Wakefield *et al*, 1987). TGF- β is normally secreted in a latent form that must be activated either by proteolytic cleavage (Wakefield *et al*, 1988) or by treatment with acid (Wadhwa *et al*, 1991).

TGF- β exhibits a diverse range of properties, directed towards most cell types, and is an important mediator of numerous cellular and physiological processes (Danielpour *et al*, 1989; Roberts and Sporn, 1990). Its biological effects depend on several factors (Wahl, 1992), namely tissue bed, target-cell type, state of cell differentiation, influence of other growth factors and latent or active TGF- β . TGF- β also inhibits the growth of many cell types in culture, including epithelial cells and hepatocytes (Muller *et al*, 1987; Takehara *et al*, 1987; Coffey *et al*, 1988), and stimulates the growth of others (Moses *et al*, 1985). It causes chemotaxis of monocytes and neutrophils (Wahl *et al*, 1987; Reibman *et al*, 1991). TGF- β causes synthesis of extracellular matrix proteins, such as collagen (Ignotz and Massague, 1986; Roberts *et al*, 1986) and fibronectin (Ignotz and Massague, 1986), and of cellular receptors for matrix proteins. As a cytokine, TGF- β is potentially important because it may induce immunosuppression (Wahl, 1992) antagonising many B and T lymphocyte responses (Kehrl *et al*, 1986a; 1986b). Hence, it inhibits T cell proliferation to polyclonal mitogens or in mixed leukocyte reactions and inhibits maturation of CTLs. On non-immune effector cells, such as PMNs and endothelial cells, TGF- β acts as an 'anti-cytokine', counteracting the effects of pro-inflammatory

cytokines. It can also inhibit macrophage activation. In summary, TGF- β is largely a negative regulator of immunity, although it has some positive effects.

Although some differences have been reported in the *in vitro* biological activities of TGF- β_1 and TGF- β_2 (i.e. TGF- β_2 lacks the capacity of TGF- β_1 to inhibit haematopoiesis) (Cheifetz *et al*, 1987; Ruscetti *et al*, 1991), in general TGF- β s 1, 2 and 3 have qualitatively similar activities when added to cells in culture (Graycar *et al*, 1989). Moreover, competition studies with TGF- β_1 , - β_2 and - β_3 also indicate that all three proteins interact with the same cell-surface binding molecules (Graycar *et al*, 1989). Whether the three isoforms have distinct biological functions *in vivo* is still unknown (Pelton *et al*, 1990).

6.2.2 TGF- β IN THE LUNG

TGF- β is one of the constitutively expressed cytokines of the normal mammalian lung (Tovey, 1988). The presence of this cytokine on epithelial surfaces of the distal lung stabilises the structural cell populations of the normal lung (Kelley, 1990). In normal human airways, TGF- β acts as a primary differentiation-inducing factor for bronchial epithelial cells (Masui *et al*, 1986). It also modulates antioxidant defense systems and the release of surfactant by epidermal growth factor (Kelley, 1990).

In contrast to its contribution to pulmonary homoeostasis, TGF- β is also involved in the biology of pulmonary disease. Hence, the constitutive levels of TGF- β in the lung can suppress intrapulmonary populations of T and B lymphocytes and NK cells (Robinson *et al*, 1984; Holt, 1986). Furthermore, high local levels of TGF- β may critically suppress repair processes after tissue injury, through the inhibition of proliferation of endothelial cells (Heimark *et al*, 1986). Some pathogenic role for TGF- β_1 in pulmonary fibrosis has also been suggested (Broekelmann *et al*, 1991).

6.3. MATERIALS AND METHODS

6.3.1 EXPERIMENTAL SHEEP

Twelve male sheep (median body weight 46 kg, range= 35-52 kg) were purchased from an accredited MVV-free Texel flock at the age of 8 months. Animals were determined to be free of serum antibody to MVV by AGIDT (Winward *et al*, 1979) at the time of purchase.

To prevent horizontal transmission of MVV, each group of animals was housed separately and direct contact between the different groups was prevented. Animals were fed *ad libitum* hay and water with a regulated supply of concentrates. Prior to BAL, animals were housed in individual pens, being moved to their communal pens shortly after recovery from the anaesthesia. The sheep were inoculated at 13 months old.

6.3.2 PREPARATION OF THE VIRAL ANTIGEN

Monolayers of ovine skin cells, derived from a skin biopsy from the groin of Finn-cross adult sheep (Bird *et al*, 1993), were grown into T-75 (75 cm²) tissue culture flasks (Costar, Cambridge, UK) in Dulbecco's modified Eagle medium (DMEM) (Gibco Ltd., Paisley, Scotland) (Appendix II.7) supplemented with 2mM L-glutamine, 100 U/ml of benzyl-penicillin, 100 U/ml of streptomycin and 8% heat-inactivated foetal calf serum (Ato Tek, Ltd., Glasbury, Hereford, UK) until 90% confluent. Skin cell monolayers were then infected with 0.1 TCID₅₀/cell of EV1 strain of MVV (Sargan *et al*, 1991) in DMEM supplemented with 2% foetal calf serum (2% DMEM). When extensive cytopathic effects were seen (usually 5-7 days post-infection), the virus-containing cell supernatant was harvested, clarified by centrifugation at 800 g for 20 min, and stored at -70°C. The titre of the virus stock was determined on skin cell monolayers, and the infectivity titre was calculated by using the quantal method of Reed and Muench (Mohanty and Dutta, 1981).

6.3.3 HEAT INACTIVATED VIRUS

The EV1 strain of MVV, prepared as described above (6.3.2), was inactivated by incubation in a water bath at 56°C for 1h. No residual infectivity remained as determined by lack of cytopathic effect when cultured in monolayers of ovine skin cells.

6.3.4 MOCK-INFECTED ANTIGEN

Monolayers of ovine skin cells were cultured, as described for the viral antigen, for 5 days. Supernatant was then harvested and clarified by centrifugation at 800 g for 20 min. This supernatant was employed as mock-infection antigen.

6.3.5 DESIGN OF EXPERIMENT

The twelve sheep were allocated to three groups. A first group (CON) of four animals was inoculated with mock-infected culture supernatant. A second group (HIC) of two animals was inoculated with heat inactivated EV1 MVV. The third group (MVV) consisting of six sheep was inoculated with $10^{7.2}$ TCID₅₀ of the strain EV1 of MVV.

6.3.6 INOCULATION OF THE ANIMALS

All the animals were inoculated via the respiratory tract. Basically, the procedure for the experimental inoculation of the animals was similar to the technique already described for collection of BALF (Chapter 2). The fiberoptic endoscope was wedged into the lobar bronchus of the left diaphragmatic lobe. 200 ml of the appropriate inoculum were then infused through the lateral channel of the endoscope. To ensure delivery of the inoculum, 25 ml of air were injected through the lateral channel of the endoscope.

6.3.7 BALF COLLECTION

BAL was performed as previously described (Chapter 2). The lobar bronchus of the left diaphragmatic lobe was always sampled. The timing of BAL procedure during the experiment is shown in Table 6.1.

Table 6.1. Timing of sampling. The first samples were collected from all animals one month prior the experimental inoculation (at the age of 12 months) to get baseline parameters.

N ^o of Sampling	Weeks PI
0*	0*
1	2
2	4
3	6
4	8
5	12
6	16

* Baseline

PI=post-inoculation

6.3.8 PERIPHERAL BLOOD (PB), PLASMA AND SERA COLLECTION

PB samples from all animals were collected simultaneously with BALF samples (Table 6.1) as previously described (Chapter 2). For routine haematology (leukocyte and erythrocyte counts, hematocrit, and leukocyte differential counts), PB was collected into evacuated tubes containing EDTA (Becton Dickinson, Rutherford, USA). For serology, blood was collected into preservative-free tubes (Becton Dickinson, Rutherford, USA) and serum was stored at -20°C. For plasma collection,

PB was collected into heparinised evacuated tubes (Becton Dickinson, Rutherford, USA).

6.3.9 ANTIBODY ASSAY

Sheep serum was examined for specific precipitating antibodies to MVV by the AGIDT (as described in Chapter 2). The first AGIDT was performed at the time of the purchase of the animals. Then, AGIDT was performed at each sampling period, and at 20 and 24 weeks PI.

6.3.10 CLINICAL EVALUATION

The inoculated sheep were monitored weekly for clinical signs of respiratory disease. Haematological examination was performed at each sampling period.

6.3.11 FLOW CYTOMETRIC ANALYSES

Single and dual colour flow cytometric analyses were performed on BALF lymphocytes as described in Chapters 3 and 4.

6.3.12 MAbs EMPLOYED

For single colour staining on BALF CD5⁺ lymphocytes, the murine MAb ST1 (Beya *et al*, 1986), with CD5 reactivity, was the only primary stage reagent used.

As second stage reagent a FITC-conjugated F(ab')₂ fragment of rabbit immunoglobulins to mouse Ig (Dako, High Wycombe) was employed at a previously determined optimal dilution of 1:100.

The MAbs and immunoconjugates employed for dual colour flow cytometric analyses of BALF lymphocytes, and their working dilutions were the same as reported in Chapter 4 (Table 4.1), except for MAb SW73.2, which was not used.

6.3.13 TGF- β GROWTH INHIBITION ASSAY

Quantification of plasma and PELF TGF- β was performed as described in Chapter 2 (section 2.15).

6.3.14 PLASMA AND BALF UREA DETERMINATIONS

Plasma and BALF urea determinations were as described in Chapter 2 (section 2.16).

6.3.15 STATISTICAL ANALYSES

Cell counts, flow cytometry data and TGF- β levels were not normally distributed.

Among group comparisons: Comparisons among the three groups were made using the non-parametric Kruskal-Wallis test, with a significance level of 95%.

Within group comparisons: For comparisons within the control and the MVV-inoculated group the paired-t test was employed with a significance level of 95%. No statistics were performed in the HIC group due to the small number of animals.

6.4. RESULTS

6.4.1 CLINICAL STATUS OF THE ANIMALS

During an observation period of 4 months, clinical and haematological examination of the three groups of animals, did not indicate the presence of disease.

During the experiment, five of the animals died after BALF samples were taken, while the fifth animal was humanely destroyed due to abomasal torsion.

6.4.2 ANTIBODY RESPONSE

Results of the AGIDT are shown in Table 6.2. Precipitating antibodies to MVV developed within the first two weeks PI in three of the MVV-inoculated animals, and between the second and the fourth week PI in the other three MVV-inoculated animals. No seroconversion was observed either in the control sheep or in the HIC group. This confirmed that all animals inoculated with MVV were successfully infected with MVV, and that segregation of control and MVV-infected sheep was effective.

Table 6.2. Development of serum precipitating antibodies to MVV as determined by the AGIDT.

Sheep n ^o	P	WEEKS PI								
		0	2	4	6	8	12	16	20	24
CON 70	-	-	-	-	-	D				
CON 107	-	-	-	-	-	-	-	-	-	-
CON 125	-	-	-	-	-	-	D			
CON 129	-	-	-	-	-	-	D			
HIC 35	-	-	-	-	D					
HIC 141	-	-	-	-	-	-	D			
MVV 14	-	-	-	+	+	+	+	+	+	+
MVV 56	-	-	-	+	+	+	+	+	+	+
MVV 95	-	-	+	+	+	+	+	+	+	+
MVV 102	-	-	+	+	+	+	+	+	+	+
MVV 105	-	-	-	+	+	+	+	+	+	+
MVV 120	-	-	+	+	+	+	+	+	+	+

P = Time of purchase

D = Death

6.4.3. BALF CELL PHENOTYPIC ANALYSES

6.4.3.1 Comparison of data for MVV-inoculated animals and other groups

Compared with the other two groups, BALF from MVV-inoculated animals had significantly ($p < 0.05$) higher percentages of lymphocytes and significantly lower percentages of BALF macrophages at 4 weeks PI (Fig 6.1, appendices I.6.1 and I.6.2).

Within the BALF lymphocyte population of the MVV-inoculated group there was a significant ($p < 0.05$) increase over control and HIC values in the percentage of (a) **CD8⁺** T cells at 2, 4, 6 and 8 weeks PI (Fig. 6.2, appendix I.6.4), and (b) **CD4⁺/VPM36⁺** T cells (MHC Class II-DQ) at 2 weeks PI (Fig. 6.4, appendix I.6.9).

When compared with the other two groups MVV-inoculated sheep showed significantly ($p < 0.05$) decreased percentages of BALF (a) **CD4⁺** T cells at 6, 8 and 12 weeks PI (appendix I.6.3), (b) decreased percentages of **CD5⁺** T cells at 6 and 8 weeks PI, (c) decreased **CD4⁺/CD8⁺ ratio** at 4, 6, 8 and 12 weeks PI, (d) $\gamma\delta$ T cells at 2, 6 and 8 weeks PI, and (e) $\gamma\delta^+$ / **VPM54⁺** T cells (MHC Class II-DR) at 4 weeks PI (Figs. 6.2 and 6.3, appendices I.6.5-I.6.8).

6.4.3.2 Within group time related changes - control animals

When compared to baseline values, control animals showed significantly ($p < 0.05$) increased percentages of BALF lymphocytes at 2, 8 and 12 weeks PI, and significantly ($p < 0.05$) decreased percentages of BALF macrophages at 2 weeks PI (Fig. 6.1).

In control animals, there were no significant changes with time in the percentages of BALF **CD4⁺**, **CD5⁺**, **CD8⁺** and $\gamma\delta$ T lymphocytes or in the **CD4⁺/CD8⁺ ratio** (Figs. 6.2 and 6.3).

Compared with baseline data, within the BALF lymphocyte population control animals had significantly ($p<0.05$) increased percentages of (a) **CD4⁺/VPM54⁺** T cells (MHC Class II-DR) at 4, 6, 8 and 12 weeks PI, (b) **CD4⁺/VPM36⁺** T cells (MHC Class II-DQ) at 6, 8, 12 and 16 weeks PI, (c) **CD4⁺/IL-2R⁺** T cells at 6, 8 ($p<0.001$) and 12 weeks PI, (d) **CD8⁺/VPM54⁺** T cells at 6, 8 and 12 weeks PI, (e) **CD8⁺/VPM36⁺** T cells at 6, 8 and 12 weeks PI, (f) **CD8⁺/IL-2R⁺** T cells at 6, 8, 12 and 16 weeks PI, (g) **$\gamma\delta^+$ /VPM54⁺** T cells at 2, 4 ($p<0.001$), 6, 8 and 12 weeks PI, (h) **$\gamma\delta^+$ /VPM36⁺** T cells at 6, 8 and 12 weeks PI, and (i) **$\gamma\delta^+$ /IL-2R⁺** T cells at 6, 8 and 12 weeks PI (Figs. 6.3-6.6).

6.4.3.3 Within group time related changes - MVV-inoculated animals

Compared with baseline values, the MVV-inoculated group showed significantly ($p<0.05$) increased percentages of BALF lymphocytes throughout the sampling period and significantly ($p<0.05$) decreased percentages of BALF neutrophils at 2 and 4 weeks PI, and percentages of BALF macrophages at 2, 4 and 6 weeks PI (Fig. 6.1).

Compared with baseline values in the lymphocyte gate, BALF from the MVV-inoculated group showed significantly ($p<0.05$) decreased percentages of (a) **CD4⁺** T cells at 4, 6, 8, and 12 weeks PI, (b) decreased **CD4⁺/CD8⁺ ratio** at 4, 6 and 8 weeks PI, (c) **CD5⁺** T lymphocytes at 4, 6 and 8 weeks PI, and (d) **$\gamma\delta$** T lymphocytes at 6 weeks PI (Figs. 6.2 and 6.3). There were, in contrast, significantly ($p<0.05$) increased percentages of (a) **CD8⁺** T cells at 2, 4 ($p<0.001$), 6 and 8 weeks PI, (b) **CD4⁺/VPM54⁺** T cells (MHC Class II-DR) throughout the sampling period, (c) **CD4⁺/VPM36⁺** T cells (MHC Class II-DQ) throughout the sampling period, (d) **CD4⁺/IL-2R⁺** T cells at 2 and 12 weeks PI, (e) **CD8⁺/VPM54⁺** T cells at 6, 8, 12 and 16 weeks PI, (f) **CD8⁺/IL-2R⁺** T cells at 8 weeks PI, (g) **$\gamma\delta^+$ /VPM54⁺** T cells throughout the study, (h) **$\gamma\delta^+$ /VPM36⁺** T cells at 4, 8, 12 and 16 weeks PI, and (i) **$\gamma\delta^+$ /IL-2R⁺** T cells at 4, 6, 8 and 12 weeks PI (Figs. 6.2-6.6).

Fig. 6.1. Time related changes in the percentages of BALF lymphocytes, macrophages and neutrophils during experimental MVV-infection. Control sheep (CON) (n=4), MVV-inoculated sheep (MVV) (n=6). Results expressed as medians.

* Significantly different between groups at $p < 0.05$

+ Significantly different from baseline at $p < 0.05$

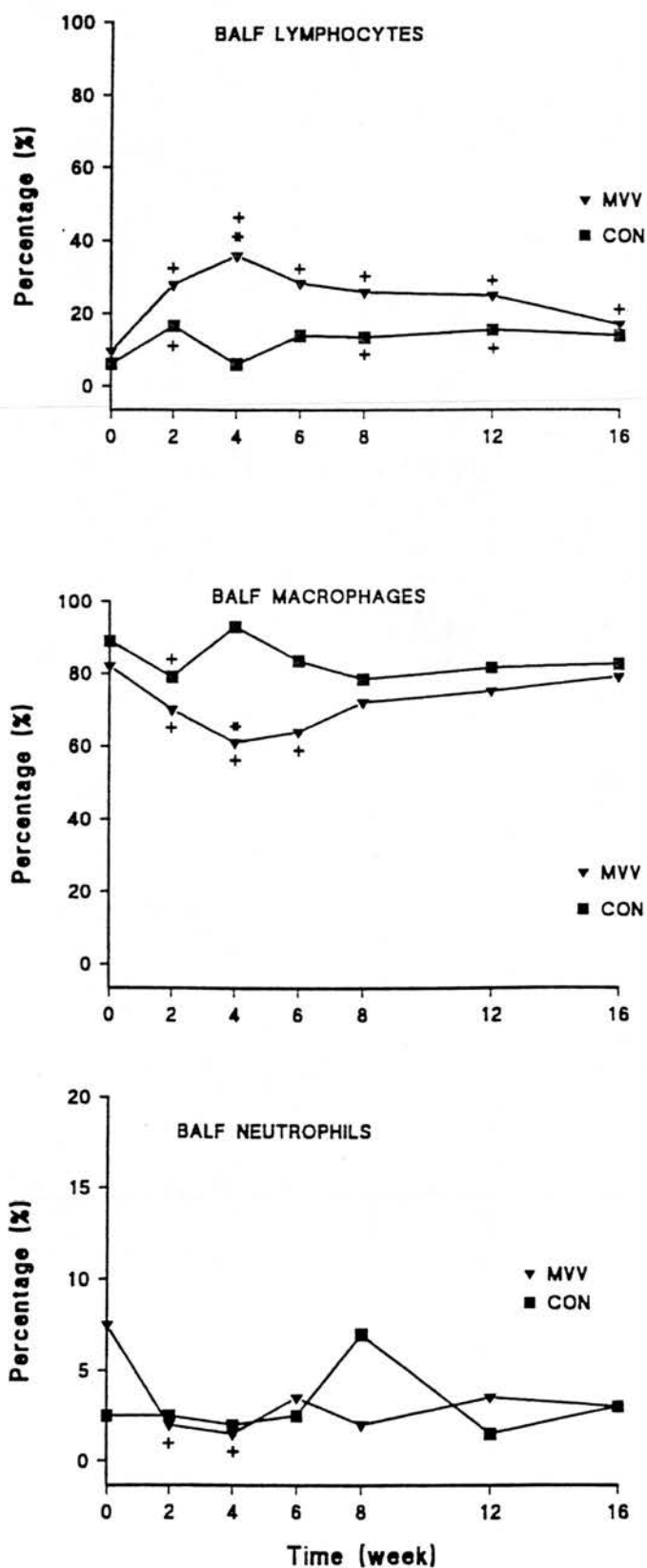


Fig. 6.2. Time related changes in the percentages of BALF lymphocytes expressing CD4⁺, CD8⁺ and CD5⁺ during experimental MVV-infection. Control sheep (CON) (n=4), MVV-inoculated sheep (MVV) (n=6). Results expressed as medians.

* Significantly different between groups at $p < 0.05$

+ Significantly different from baseline at $p < 0.05$

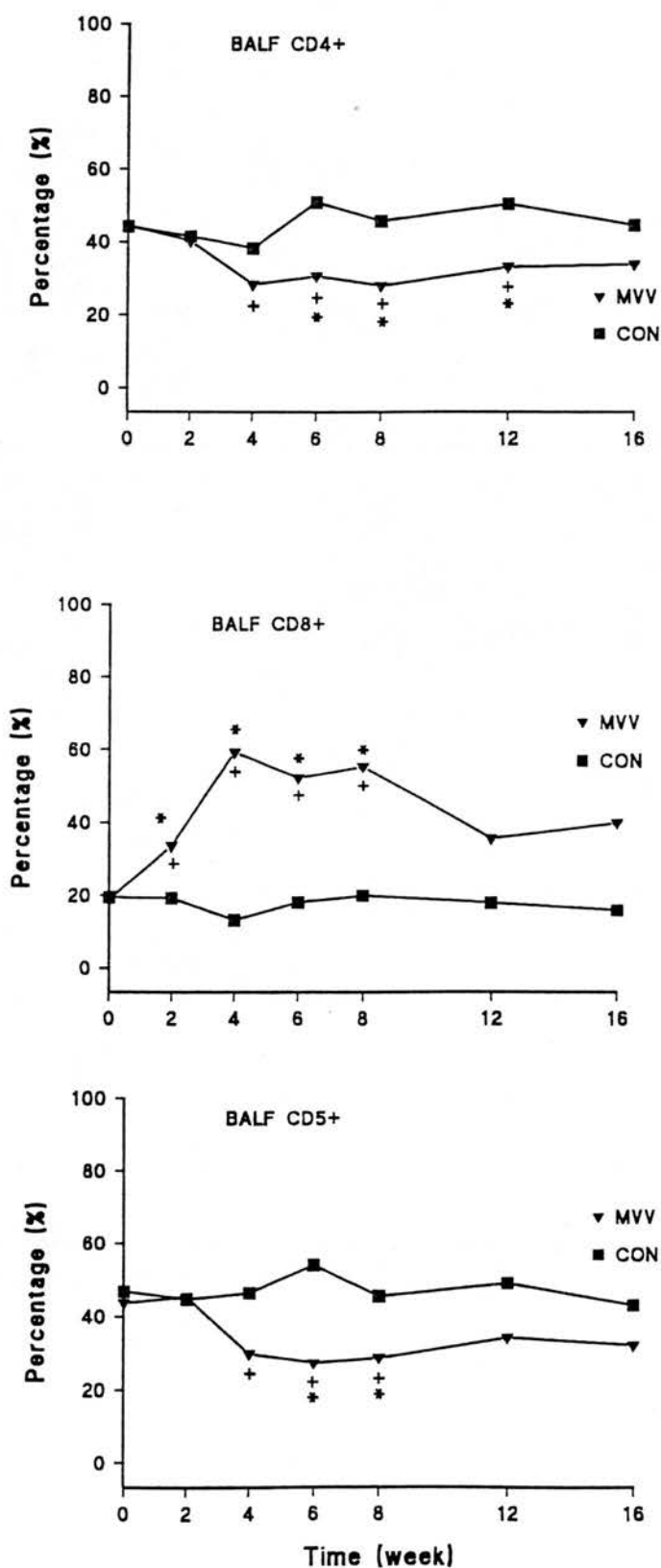


Fig. 6.3. Time related changes in the BALF CD4⁺/CD8⁺ ratio and in the percentages of BALF lymphocytes expressing $\gamma\delta^+$ and CD4⁺/VPM54⁺ during experimental MVV-infection. Control sheep (CON) (n=4), MVV-inoculated sheep (MVV) (n=6). Results expressed as medians.

* Significantly different between groups at $p < 0.05$
+ Significantly different from baseline at $p < 0.05$

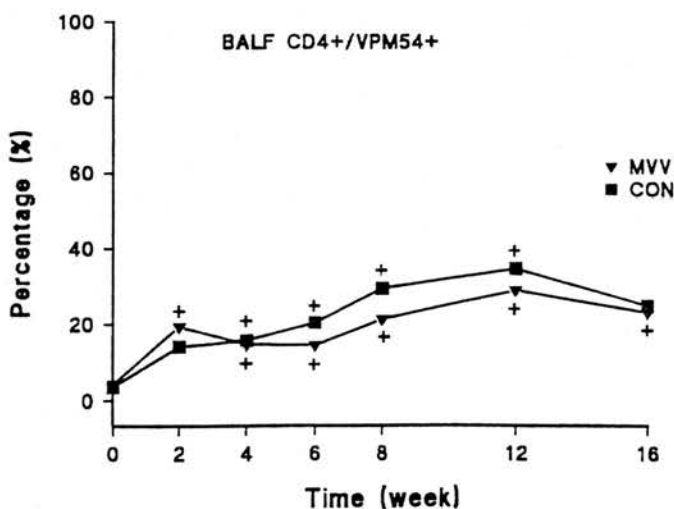
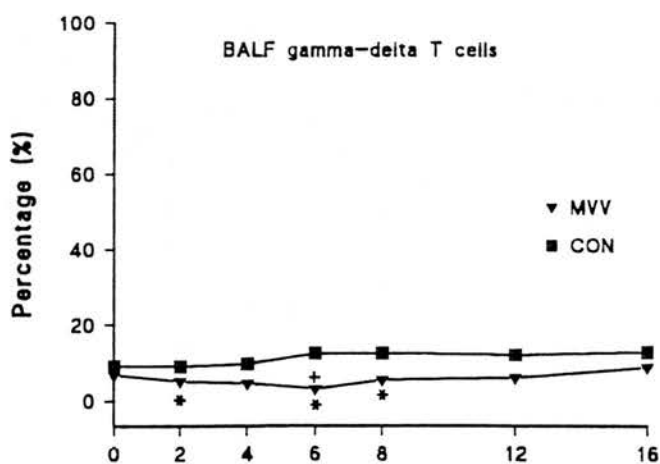
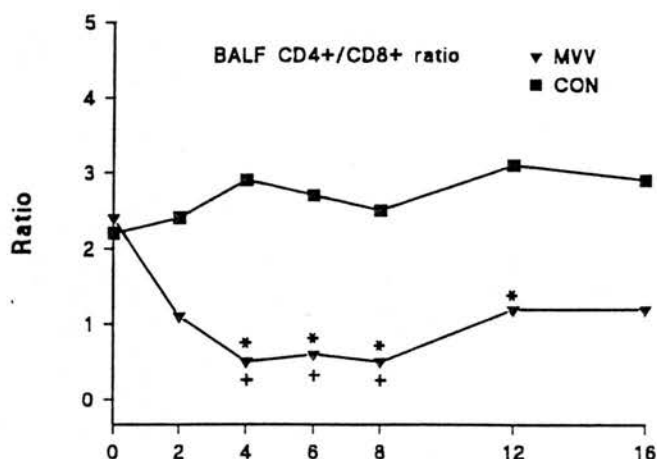


Fig. 6.4. Time related changes in the percentages of BALF lymphocytes expressing CD4⁺/VPM36⁺, CD4⁺/IL-2R⁺ and CD8⁺/VPM54⁺ during experimental MVV-infection. Control sheep (CON) (n=4), MVV-inoculated sheep (MVV) (n=6). Results expressed as medians.

* Significantly different between groups at p< 0.05

+ Significantly different from baseline at p< 0.05

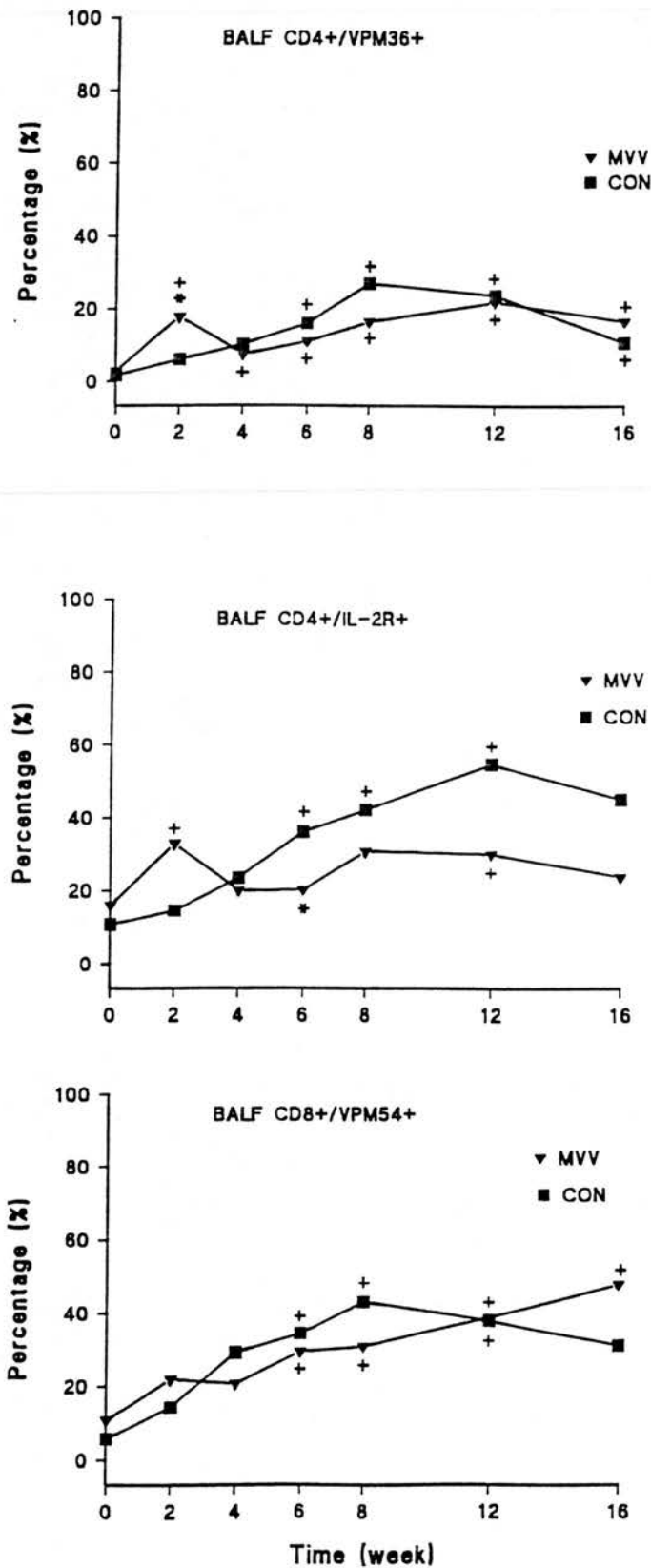


Fig. 6.5. Time related changes in the percentages of BALF lymphocytes expressing CD8⁺/VPM36⁺, CD8⁺/IL-2R⁺ and $\gamma\delta$ ⁺/VPM54⁺ during experimental MVV-infection. Control sheep (CON) (n=4), MVV-inoculated sheep (MVV) (n=6). Results expressed as medians.

* Significantly different between groups at $p < 0.05$
+ Significantly different from baseline at $p < 0.05$

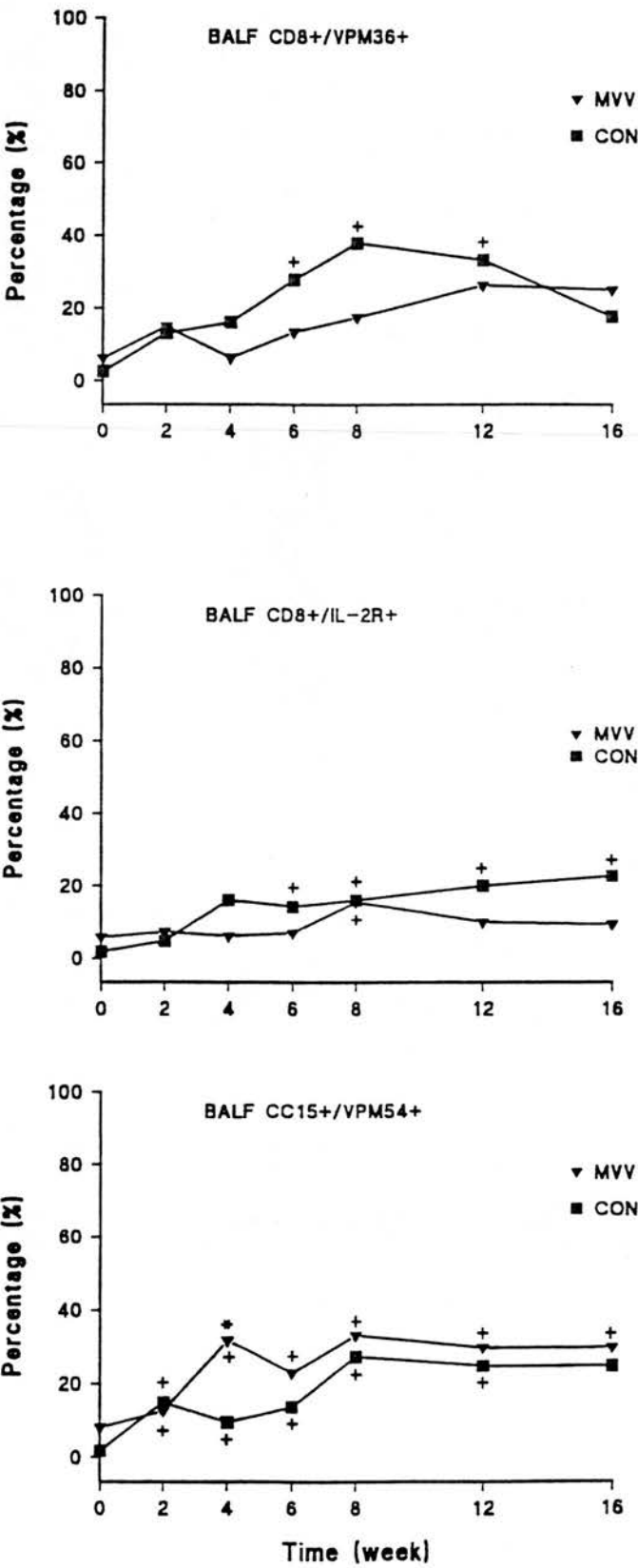
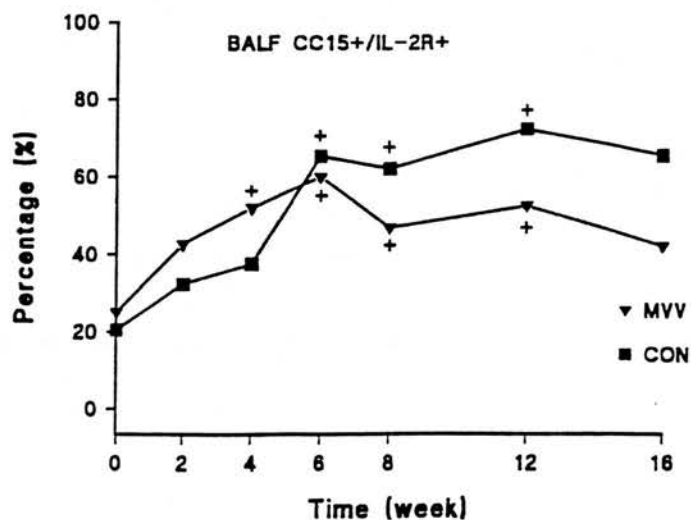
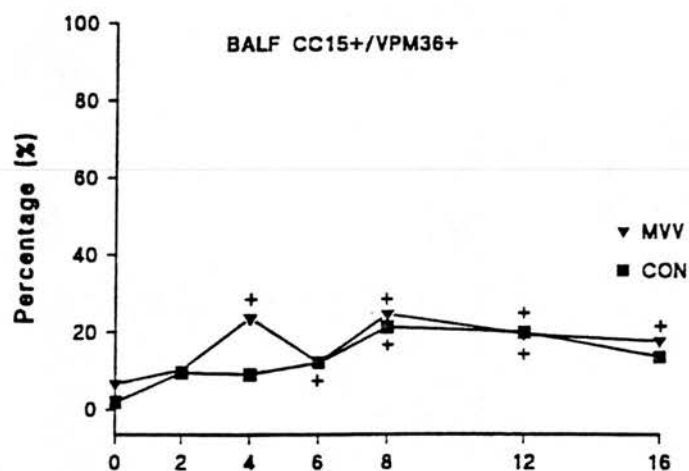


Fig. 6.6. Time related changes in the percentages of BALF lymphocytes expressing $\gamma\delta^+$ /VPM36 $^+$ and $\gamma\delta^+$ /IL-2R $^+$ during experimental MVV-infection. Control sheep (CON) (n=4), and MVV-inoculated sheep (MVV) (n=6). Results expressed as medians.

* Significantly different between groups at $p < 0.05$

+ Significantly different from baseline at $p < 0.05$



6.4.4 PLASMA AND BALF UREA

Plasma urea (Appendix I.6.17) concentrations for all animals (median 34.93 mg/dl, range 13.25-54.21) were within the working range of the assay (0-100 mg/dl). All BALF samples had urea concentrations (median 0.59 mg/dl, range 0.07-1.28) within the linear range of the assay (0.01-1.30 mg/dl) (Appendix I.6.17).

Intraassay coefficient of variation for urea determination of BALF samples with low (median 0.24 mg/dl) and high (median 1.16 mg/dl) urea concentrations, based on 5 repeated duplicate assays were 0.43% and 0.54% respectively.

6.4.5 TGF- β BIOASSAY

In this assay, the inhibitory concentration (IC_{50}), or concentration of either TGF- β_1 or TGF- β_2 required for half maximal inhibition of proliferation of MV.1.Lu mink lung epithelial cells was approximately 0.06 ng/ml (Fig. 2.7). Lack of reagents for TGF- β_3 precluded investigation of this homodimer.

Both TGF- β_1 and TGF- β_2 neutralising antibodies partially blocked BALF supernatant and plasma induced inhibition of MV.1.Lu mink lung epithelial cells proliferation (Table 6.3). Acid activated BALF supernatant and plasma that had been incubated with anti-TGF- β antibodies did not inhibit DNA synthesis by MV.1.Lu mink lung epithelial cells to the same extent as acid-activated BALF supernatant and plasma alone. Nonimmune chicken IgG₁ did not alter the inhibition. This indicates that most of the inhibition in BALF and plasma was due to TGF- β_1 and TGF- β_2 . These results demonstrate the usefulness of the assay in estimating TGF- β levels in unfractionated conditioned media that may contain an undefined mixture of growth factors.

Table 6.3. Blocking of TGF- β_1 and TGF- β_2 -induced inhibition of MV.1.Lu mink lung epithelial cells proliferation by preimmune antibody or chicken antibodies against TGF- β_1 and TGF- β_2 . Data are expressed as percent of proliferation inhibited by TGF- β_1 or TGF- β_2 . The concentration of TGF- β_1 and TGF- β_2 employed was 1 ng/ml. Anti-TGF- β_1 and anti-TGF- β_2 MAbs were used in a 1:100 dilution of the stock. As control, 3.5×10^4 cells MV.1.Lu mink lung epithelial cells in a volume of 100 μ l run without any TGF- β were employed.

SAMPLE	% INHIBITION
Control	0%
+TGF- β_1	89%
+TGF- β_2	78%
+TGF- β_1 + α TGF- β_1	1%
+TGF- β_2 + α TGF- β_2	3%
+ α TGF- β_1	0%
+ α TGF- β_2	0%
+ α IgG	0%
+TGF- β_1 + α IgG ₁	87%
+TGF- β_2 + α IgG ₁	76%
+BALF	68%
+BALF+ α TGF- β_1	7%
+BALF+ α TGF- β_2	0%
+plasma	74%
+plasma+ α TGF- β_1	11%
+plasma+ α TGF- β_2	3%

6.4.5.1 PLASMA TGF-β

No significant differences among groups were observed in the levels of plasma TGF-β. No significant time-related changes were observed within groups (Fig. 6.7 and Appendices I.6.18 and I.6.21).

6.4.5.2 PELF TGF-β

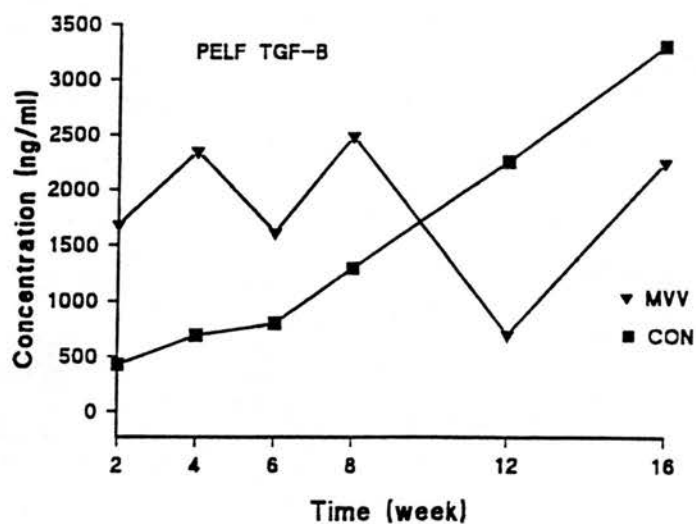
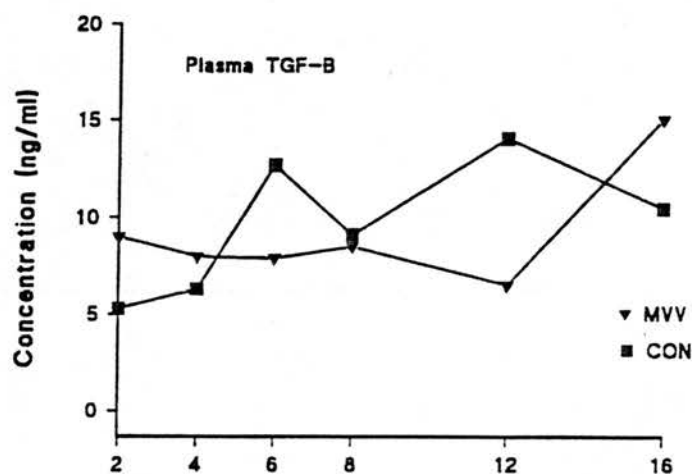
Concentrations of TGF-β in PELF, as derived from BALF TGF-β concentrations by the 'urea dilution technique' (Chapter 2) are shown in Appendices I.6.20 and I.6.22. The BALF concentrations, which were used to calculate the PELF TGF-β are given in Appendix I.6.19.

No significant differences in the levels of PELF TGF-β either among or within groups were observed (Fig. 6.7, appendix I.6.22). PELF contained approximately 165-fold more TGF-β₁ than did plasma from the same animals (Table 6.5).

Table 6.5. Concentrations of TGF-β (ng/ml) in plasma and in PELF for: **ALL**= combined data for three groups of animals; **CON**=control group; **HIC**= group inoculated with heat inactivated MVV; **MVV**= group inoculated with MVV (median of concentrations of TGF-β at different sampling times and range).

	TGF-β (ng/ml)	
	PLASMA	PELF
ALL	9.1 (0.4-29.6)	1167.0 (255.0-5736.0)
CON	10.1 (0.4-29.6)	1038.0 (255.0-5736.0)
HIC	9.3 (6.6-14.3)	776.2 (582.4-1131.7)
MVV	8.2 (0.4-19.9)	2027.0 (369.0-5650.0)

Fig. 6.7. Time related changes in plasma and PELF TGF- β during experimental MVV-infection. Control sheep (CON) (n=4), MVV-inoculated sheep (MVV) (n=6). Results are expressed as median of concentrations (ng/ml).



6.5. DISCUSSION

Natural and experimental MVV infection show the typical pattern of a slow virus infection regarding appearance of clinical signs and development of pathological lesions (Sigurdsson, 1954). Virus persists for a very long time in the animal despite the presence of active cellular and humoral immune responses (Larsen *et al*, 1982a). This study of the early cellular immune response in the sheep lung following inoculation with MVV, shows that an acute cellular immune response occurs in the lung within 4 weeks PI.

A model was developed in which a large inoculum of MVV was delivered by bronchoscopy to a defined segment of one lobe of a sheep's lung with the expectation that most of the changes of interest would take place in a small and defined area. BALF was then collected at periodic intervals for 4 months. This model allowed ready access to the target tissue and subsequent sequential studies in individual animals. It was also expected that the large experimental inoculum would reproduce the pulmonary disease soon after the initial infection. The main disadvantage of the present work was that, in order to collect BALF, sheep had to undergo general anaesthesia every two weeks. Anaesthesia, induced with intravenous thiopentone sodium, was not always well tolerated, and four of the animals died either during anaesthesia or during the anaesthetic recovery process.

The success of the experimental MVV infection in this work was confirmed by the appearance of precipitating antibodies to MVV in the six sheep inoculated with the virus between 2 and 4 weeks PI. No seroconversion was observed in the other two groups of animals. This is concordant with previous results in MVV experimentally inoculated sheep, where precipitating antibodies were found within a few weeks to a few months after inoculation of large doses of virus (Terpstra and De Boer, 1973; Oliver *et al*, 1981b; Larsen *et al*, 1982a; Tuboly *et al*, 1991). These antibodies are

thought to remain detectable in the blood for years (De Boer, 1975), and probably throughout the life of the sheep. The sampling period in this work was not long enough to assess for how long precipitating antibodies persist in blood.

During the 4 month sampling period no clinical signs were detected in the MVV-infected animals. The absence of clinical signs was expected considering that the incubation period to clinical disease is highly irregular, ranging from 2 months to over 10 years in experimentally infected sheep (Nathanson *et al*, 1976).

When compared with control and HIC animals, MVV-inoculated sheep presented significantly increased percentage of BALF lymphocytes and significantly decreased percentage of BALF macrophages 4 weeks PI. However, both control and MVV-inoculated animals presented increased percentages of BALF lymphocytes and decreased percentages of BALF macrophages, when compared with baseline values, as soon as 2 weeks PI. Increased numbers and percentages of BALF lymphocytes (Lairmore *et al*, 1986; 1988a; Cordier *et al*, 1992) and neutrophils (Cordier *et al*, 1992) in experimentally MVV-infected animals have been described. However, all previous studies were performed on excised lungs from lambs inoculated transtracheally with MVV. No previous data on the BALF cellular composition of MVV-experimentally infected adult sheep are available. Furthermore, in previous studies the sacrifice of the animals did not permit repeated sampling during the experiment.

The finding of increased percentages of BALF lymphocytes in the three groups of animals suggests that this increase is not directly induced by MVV. An inflammatory response induced by repeated BAL would also be unlikely, since it was shown in a previous study that BAL can be repeated every two weeks without influence on cellular or humoral contents of ovine BALF (Begin *et al*, 1981). It is

possible that recruitment of lymphocytes from PB or from lymphoid organs is a response to the inflammatory effect caused by the inoculum itself, which contained 10% foetal calf serum. The existence of an inflammatory response in the lung as result of its inoculation with MVV has been described and grossly characterised (Staskus *et al*, 1991). In this work, each animal was inoculated with a 200 ml suspension of tissue culture supernatant. Any constituent of the tissue culture medium employed to prepare these suspensions could have acted as an antigen, causing inflammation at the inoculation site and, therefore, accounting for the increased percentage of lymphocytes in the three groups of animals.

As the experimental infection progressed, in BALF from MVV-inoculated animals there was a significant decrease in the percentages of $CD4^+$, $CD5^+$ and $\gamma\delta$ T cells, an increase in the percentage of $CD8^+$ T cells and inversion of the $CD4^+/CD8^+$ ratio. These findings are analogous to those from a previous study in MVV-infected sheep (Lairmore *et al*, 1988a), where the $CD8^+$ subset was the predominant T cell subset in BALF. These results, however, contradict those of Cordier and others (1992), who reported an equal increase in the proportion of BALF $CD4^+$ and $CD8^+$ T cells with no inversion of the $CD4^+/CD8^+$ ratio. However, while Lairmore and others (1988a) inoculated their control sheep with virus-free culture medium alone as was done in the present study, the study of Cordier and others (1992) was not properly controlled as control sheep were inoculated with saline. Whether the changes in the proportions of the different lymphocyte subsets are due to a migration of those lymphocyte subpopulations to blood or other lymphoid organs, or to a continuous destruction and regeneration can not be elucidated from this study.

The decreased percentage of BALF $\gamma\delta$ T cells in the MVV-inoculated animals could indicate participation of these cells in the pulmonary cellular immune response as suggested by Agostini and Semenzato (1990). Interestingly, in the present study, $\gamma\delta$

T cells were the first T cell subset to return to baseline values in the MVV-infected group. Although no changes in the pulmonary $\gamma\delta$ T cells were observed in natural MVV-infection (Lujan *et al*, 1993), the present data suggest participation of these cells in the early pulmonary immune response.

In the present study, a very acute cellular immune response, detected by phenotypic changes in BALF T cell subsets, was mounted against the MVV inoculum. Early and short duration (2-6 weeks) cellular immune responses were also demonstrated in experimentally induced MVV infection using the lymphocyte transformation test (Griffin *et al*, 1978; Larsen *et al*, 1982a). However, despite using a high titre viral inoculum, lymphocyte phenotype changes returned to normal within 2 or 3 weeks. Several explanations can be proposed to account for the short duration of the cellular immune response in the MVV-inoculated animals. Firstly, it is possible that the infectivity of the MVV used, although sufficient to induce cytopathic effect *in vitro*, was too low to induce lymphoproliferative disease and widespread virus distribution *in vivo*. The cytopathic effect of high passage, laboratory adapted strains of lentiviruses do not accurately predict *in vivo* pathogenicity (Lairmore *et al*, 1987). Secondly, the inoculated MVV could have remained in a period of latency (Narayan, 1990). Thirdly, subsequent development of virus-induced immunosuppression, as occurs in infections with other retrovirus including murine leukemia virus (Friedman, 1975) and equine infectious anemia virus (Kono *et al*, 1978), could account for the observed short lived cellular immune response.

Following experimental inoculation of the sheep, the expression of MHC Class II antigens and IL-2R by BALF CD4⁺, CD8⁺ and $\gamma\delta$ T cells increased progressively in control and MVV-inoculated animals. Significant differences in the expression of MHC Class II and IL-2R antigens by T cell subsets between control and MVV-inoculated animals were short-lived (Figs. 6.4 and 6.5). Previous studies (Lairmore *et*

al, 1988a; Cordier *et al*, 1992) found no changes in the proportions of BALF T cells expressing MHC Class II antigens in either control or MVV-infected lambs. The differences in these studies could be due to differences in sampling times. The increased expression of activation markers on BALF T cells from the three groups of animals was probably induced by antigens in the culture media used to prepare all the inocula (as suggested for the increased percentage of BAL lymphocytes). These antigens could initiate the cytokine cascade and upregulate MHC Class II molecules and IL-2R expression.

As the bioassay used for TGF- β is highly sensitive (Cheifetz *et al*, 1987; Ikeda *et al*, 1987), samples could be assayed without prior concentration, avoiding substantial losses of active TGF- β that may occur during concentration (Danielpour *et al*, 1989). This bioassay, however, could not distinguish between TGF- β_1 and TGF- β_2 , as there were no differences in (a) the inhibitory effects of TGF- β_1 and TGF- β_2 on the epithelial cell proliferation assay, and (b) in the ability of antibodies against TGF- β_1 and TGF- β_2 to neutralise the inhibitory effect of BALF and plasma on epithelial cell proliferation. Furthermore, no reagents for the determination of TGF- β_3 were available. Distinction among homodimers was not considered important for this study as all them have almost identical biological actions (Cheifetz *et al*, 1987; Graycar *et al*, 1989).

This is the first study to demonstrate that TGF- β is a normal constituent of ovine PELF and plasma. Previously, high concentrations of latent TGF- β were found in lymph draining from normal ovine lungs (Perkett *et al*, 1991). PELF from all three groups of animals contained approximately 165-fold higher concentrations of TGF- β than plasma, consistent with previous studies on healthy men (Yaumachi *et al*, 1988). These findings suggest significant local production of TGF- β (Tovey, 1988; Yaumachi *et al*, 1988).

Unfortunately, baseline values for plasma and PELF TGF- β were not determined (Fig. 6.7). After 2 weeks PI, there were no significant differences in the levels of plasma or PELF TGF- β among the three groups of animals. These results indicate that dysregulation of this cytokine in PELF and plasma is not a feature of the early immune response following experimental MVV infection. However, concentrations of TGF- β in other pulmonary compartments, such as the pulmonary interstitium and vascular walls, may have been altered. Indeed, altered levels of TGF- β could account for many of the pulmonary pathological features associated with MVV. As the three isoforms of TGF- β were not distinguished, alterations in the relative concentrations of each isoform may have occurred without affecting the overall TGF- β concentration. *In situ* hybridisation and immunohistochemistry could be used to investigate the expression of TGF- β_1 , - β_2 and - β_3 mRNA in alveolar macrophages, as was done for TGF- β_1 mRNA in human pulmonary fibrosis (Broekelmann *et al*, 1991).

TGF- β_1 is present in brain tissues of humans with AIDS and increased concentrations of TGF- β are found in supernatants of PBMCs from AIDS patients. In AIDS patients, TGF- β may cause impaired immune function, depletion of CD4⁺ T lymphocytes, CD4⁺/CD8⁺ ratio inversion by promoting naive T cell growth and suppressing mature CD4⁺ T cell proliferation, and enhanced cerebral recruitment of HIV-1 infected monocytes, facilitating viral spread to and within the central nervous system (Kekow *et al*, 1990; Wahl *et al*, 1991; Wahl, 1992).

In this study, it was necessary to express TGF- β values relative to a component that would reflect the degree of dilution of the BALF. The proportion of PELF harvested by BAL is affected by several factors including the 'dwell time' (the time the lavage fluid is in contact with the pulmonary epithelium), the composition and volume of the lavage fluid, the site of BAL and the presence of pulmonary disease (Davis *et al*, 1982; Pingleton *et al*, 1983; Dohn and Baughman, 1985).

To overcome the problem of variable PELF recovery which occurs during BAL, Rennard and others (1986) proposed using endogenous urea as a tracer molecule. Urea is a small molecule which diffuses readily throughout body fluids. Assuming that the concentration of urea in PELF is the same as that of plasma, simultaneous determinations of plasma and BALF urea permit standardisation of the variable concentrations of PELF in BALF samples and, hence, determination of the concentration of cells and molecules in PELF. This 'urea dilution technique' was used to determine PELF TGF- β concentrations in this study. However, potential problems limit the accuracy of the urea dilution technique. Urea rapidly enters BALF during the lavage procedure (Peterson *et al*, 1993), leading to an overestimation of the volume of PELF recovered. As the magnitude of this error is proportional to the 'dwell time', in this study, BALF was aspirated immediately after instillation. Furthermore, reduced BALF recovery volumes with increased BALF urea concentrations have been described in patients with interstitial lung disease, leading also to an overestimation of PELF volume (Marcy *et al*, 1987). In view of the limitations of the 'urea dilution technique', the possibility of overestimation of the PELF volume must be considered, in which case PELF TGF- β concentrations might have been subsequently underestimated.

Other endogenous markers of BALF dilution, namely albumen, potassium and calcium and exogenous markers of dilution (i.e. markers which are incorporated to the lavage solution before BAL), such as the methylene blue dilution technique, have been used. Unfortunately, the accuracy of all these methods is limited (Rennard *et al*, 1986). Urea was considered to be a better reference marker than albumin for BALF studies in patients with interstitial lung disease (Jones *et al*, 1990). After completion of this study, a modification of the urea dilution technique which provides superior accuracy in the measurement of PELF solute concentrations was reported (Peterson *et al*, 1993).

CHAPTER 7: FINAL DISCUSSION

MVV infection, similarly to other lentiviral infections of humans and animals, is characterised by slow disease progression despite the presence of a sustained virus-specific immune response (Narayan and Clements, 1990b). The aim of this study was to further elucidate the cell-mediated immunological response at the bronchoalveolar level during the course of MVV-induced interstitial pneumonia. Although *in vitro* models have proved useful for the study of the immune response, they do not always reflect accurately what happens *in vivo*. Hence, while in tissue culture, MVV rapidly replicates to high titers, being cytolytic and causing cell fusion (Thormar, 1963), MVV infection *in vivo* is characterised by restricted viral replication (Narayan *et al*, 1977; Haase, 1986). This work, therefore, was designed to study natural MVV infection employing both *in vitro* and *in vivo* systems. Furthermore, as the early stages of the lentivirus-host interactions may be critical for disease establishment and progression (Bird *et al*, 1993), this study also investigated the early pulmonary cellular immune response during MVV infection by inducing the disease experimentally.

During the course of natural and experimental MVV infection, a series of immunologic alterations takes place in BALF cells of infected sheep (chapters 3 and 6). These changes appear soon after experimental inoculation of the animals with MVV by the respiratory route, and are mainly characterised by increased proportions of lymphocytes and neutrophils, and by phenotypic alterations in T lymphocyte subsets despite MVV not being a T-cell tropic retrovirus (Gendelman *et al*, 1985; Gorrell *et al*, 1992). The main changes in BALF T lymphocyte markers include decreased proportions of CD4⁺ and CD5⁺ T cells, increased proportion of CD8⁺ T cells, and an inverted CD4⁺/CD8⁺ ratio (chapter 6). Lymphocyte function, as determined by *in vitro* studies (chapter 5), is also compromised during MVV infection. Interestingly, T cell behaviour during MVV infection is independent of the amount of viral antigen, as extensive lymphocytic responses unrelated to the number

of cells productively infected with the virus, have been observed during MVV infection (Watt *et al*, 1992). This suggests that soluble mediators in the lung regulate the T cell response, rather than the virus *per se*.

Maedi is an interstitial lung disease. Interstitial lung diseases are a group of heterogeneous, chronic disorders that involve the entire lung parenchyma as well as the alveolar interstitium (Spencer, 1977; Fulmer and Crystal, 1979). The term 'interstitial' was originally applied to these disorders because they are associated with thickening of the alveolar septum. However, it is important to emphasise that interstitial lung disorders are not confined to the alveolar interstitium, as they also involve alveolar epithelial and endothelial cells, and even airways, arteries and veins (Crystal *et al*, 1981). The initial manifestation of these disorders is an alveolitis, defined by accumulation of inflammatory and immune effector cells within the alveolar structures (Barry *et al*, 1979). The alveolitis in interstitial lung disease produces a derangement of the alveoli, ultimately leading to loss of functional gas exchange. This alveolitis is maintained by the local production of specific cell-derived chemotactic factors that recruit inflammatory cells from the blood into the alveolar interstitium (Crystal *et al*, 1981).

In common with other interstitial lung diseases, the earliest manifestation of pulmonary MVV-infection is a chronic inflammatory process within the alveolar structures. To characterise and quantify this alveolitis, the recovery of the inflammatory and immune effector cells involved was necessary. The validity of the BAL technique as a research tool for the study of the pulmonary inflammatory component during MVV-infection can be easily deduced from these studies. In this work, not only did the BAL procedure allow the recovery of BALF for further immunological studies of its cells and soluble proteins, but also made possible the follow-up of the early cellular immune response in the lung of animals experimentally

inoculated with MVV. Since BAL in sheep can be repeated numerous times during the course of lung disease (Begin *et al*, 1981), this technique is a valuable clinical and research tool which permits an immediate approach to maedi by assessment of the ongoing alveolitis.

During natural MVV-infection, a local inflammatory response with cellular phenotypic changes occurs in the alveoli without detectable concomitant changes in peripheral blood cells. Absence of correlation between PB and BALF lymphocyte phenotypes has also been reported in healthy humans (Yamada *et al*, 1986) and during the course of several lung diseases, including pulmonary sarcoidosis (Pinkston *et al*, 1983; Muller-Quernheim *et al*, 1986; 1992) and hypersensitivity pneumonitis (Mornex *et al*, 1984). The differences between lung and blood lymphocyte populations could partially explain this divergence. Hence, lung lymphocytes have different migratory properties from PB lymphocytes (Reynolds, 1978) and they seem to be in a continuous state of activation (Semenzato, 1991). The tropism of MVV for the lung (Narayan *et al*, 1988b) may also partly explain the pulmonary compartmentalisation of the immune response. Therefore, previous studies have shown that, while PB monocytes in sheep persistently infected with MVV have viral genomes which express virus specific products minimally (Narayan *et al*, 1983; 1984), more alveolar macrophages of MVV-infected animals contain the viral genome, although only a few of these macrophages produce infectious virus (Narayan and Cork, 1985; Staskus *et al*, 1991).

Comparisons of inflammatory and immune effector cells from BALF and from open lung biopsies of patients with interstitial lung diseases have demonstrated that cells and proteins derived from the alveolar epithelial surface accurately reflect the effector components within the alveolar interstitium (Hunninghake *et al*, 1979). However, in this study while BALF from MVV-infected animals had increased

numbers of lymphocytes, similar to bronchus-associated lymphoid tissue (BALT) and alveolar septae (Watt *et al*, 1992), when phenotypic changes of BALF lymphocytes of MVV-infected animals were compared with those reported in alveolar interstitium (Watt *et al*, 1992), some differences were found. Hence, the proportion of CD4⁺ lymphocytes and the CD4⁺/CD8⁺ ratio were reduced in BALF from MVV-infected animals, whereas, in alveolar interstitium, inversion of the CD4⁺/CD8⁺ ratio and decreased proportions of CD4⁺ lymphocytes were not observed. These results suggest a further compartmentalisation of the lymphocyte-mediated immune response within the lung (Berman *et al*, 1990). Lymphocyte phenotypic alterations in response to MVV infection might occur initially within the lung parenchyma with subsequent recruitment of specific T cell subsets into the alveoli. In healthy lung, entry of lymphocytes from the blood into the lung parenchyma, and the later recruitment of specific populations of pulmonary interstitium lymphocytes into the bronchoalveolar milieu involve a series of defined steps. The two main steps are, firstly, adherence of blood lymphocytes to endothelia and secondly, induction of a motile response which permits subsequent migration of lymphocytes through the lung tissue. These steps require specific lymphocyte-endothelial and lymphocyte-chemoattractant interactions that are modulated by the local production of regulatory factors, such as cell adhesion molecules LFA-1 and LFA-3, at the site of inflammation (Pardi *et al*, 1992). The fact that LFA-1 and LFA-3 molecules were upregulated on BALF lymphocytes from MVV-infected animals (chapter 3) could account for the increased recruitment of lymphocytes to the bronchoalveolar structures in maedi. Cytokines produced by activated T lymphocytes, such as γ -IFN (Duijvestijn *et al*, 1986) might also regulate the migration of specific T cell subsets into the alveoli.

As inflammatory and immune processes within the alveolar structures can operate independently of the rest of the body (Hunninghake *et al*, 1979), and even independently in the different lung compartments (Berman *et al*, 1990), the

importance of direct assessment of the BALF itself to monitor the evolution of MVV infection at the bronchoalveolar level can be seen.

The proportion of BALF CD4⁺ lymphocytes is reduced during lentiviral infection. Whether this is due to reduced migration of CD4⁺ lymphocytes from the pulmonary interstitium into the airways and alveoli, or to destruction of this cell type in the bronchoalveolar milieu associated with a normal migration rate, is unknown. If the first postulate is correct, the presence of normal numbers of CD4⁺ lymphocytes in the pulmonary interstitium, despite the reduced numbers of CD4⁺ lymphocytes in BALF, could explain the lack of a profound immunosuppression in MVV-infected sheep. This is an important difference with respect to HIV-infection, where a complete depletion of CD4⁺ cells is present (Agostini *et al*, 1993). If the second postulate is assumed, destruction of BALF CD4⁺ lymphocytes could be caused by BALF CD8⁺ cytotoxic lymphocytes. In HIV infection, since the virus is tropic for CD4⁺ cells, the principal role of the CD8⁺ cytotoxic suppressor cell is to target the CD4⁺ lymphocyte and possibly mediate its destruction (Rankin *et al*, 1988). During MVV-infection, unlike in HIV infection, CD4 does not act as the cellular receptor for MVV (Gorrell *et al*, 1992). The mechanism that would induce CD8⁺ cytotoxic cells within the MVV-infected lung to attack CD4⁺ T cells is not well understood, but it is possible that lymphocytes could bind virus particles and such virus-bearing cells would be targeted either specifically by cytotoxic T cells, or in a non-specific or antibody-dependent manner by lymphokine-activated killer cells, NK cells or infected macrophages (Gorrell *et al*, 1992). An activation-induced apoptosis of both T cell subsets, as has been recently observed in HIV infection (Cohen *et al*, 1993), can not be ruled out. In this case, differences in the rate of cell replacement between CD4⁺ and CD8⁺ T cells can explain the decrease in the BALF CD4⁺/CD8⁺ ratio. In HIV infection, it has been suggested that an increase in the level of activation markers could account for the decline of CD4⁺ population (Mahalingam *et al*, 1993). In BALF of MVV-infected

animals expression of activation markers is equally increased on both CD4⁺ and CD8⁺ T lymphocytes, while only CD4⁺ T lymphocytes are diminished. Once more, the only way to explain this would be by assuming a different replacement rate for CD4⁺ and CD8⁺ T cells.

The augmentation of the proportion of BALF CD8⁺ T cells could be the result of a specific proliferative response against MVV-infected cells. Pulmonary macrophages, latently infected or expressing viral protein on their membrane, could induce the recruitment and/or the *in situ* expansion of CD8⁺ T lymphocytes, selectively directed against the infected cell. This would account for the 'lymphocytic alveolitis' that is present from the early phase of the disease. The proliferative response of BALF CD8⁺ T cells could be a defence mechanism following previous reduction of this T cell subset caused either by direct destruction by MVV-infected macrophages or by activation-induced apoptosis as suggested above. Virus-specific CD8⁺ T lymphocytes represent a major host defence mechanism in viral diseases in clearing virus-infected cells (Meignan *et al*, 1990). The putative intrapulmonary immunologic conflict between CD8⁺ T cells and MVV-infected alveolar macrophages could lead to immunologically induced inflammation and tissue damage. CD8⁺ T lymphocytes suppress *in vitro* replication of HIV in PBMCs (Walker *et al*, 1986). This antiviral activity appears to be mediated in part by a diffusible factor which will act interfering with virus replication (Walker and Levy, 1989; Mackewicz and Levy, 1992), and it has also been reported in studies with simian immunodeficiency virus (SIV) (Kannagi *et al*, 1988) and in HIV-1 infected chimpanzees (Castro *et al*, 1991). Unfortunately, in spite of evaluating a wide range of cytokines, attempts to identify and characterise this lymphokine produced by CD8⁺ T cells have not been successful. Brinchmann and others (1991) reported that TNF α and IFN α , β and γ , are not responsible for this anti-HIV activity of CD8⁺ cells, based on the inability of MAbs specific for these cytokines to inhibit the antiviral activity of CD8⁺ cells. Studies in

experimental models have shown that cytotoxic T lymphocytes, although capable of mediating virus clearance can also induce deleterious effects in the lung infected by the virus (Meignan *et al*, 1990).

The increased proportion of CD8⁺ T lymphocytes in lentiviral infected sheep could also account for the slow replication of MVV in tissues. Slow replication of MVV in the host alveolar macrophages could help the virus to elicit immunological responses, favouring its persistence in the host. Induction of CD8⁺ T lymphocytes in a defective maturation state is a common feature of the immune response to lentiviruses (Salazar-Gonzalez *et al*, 1985). Hence, another possibility is that CD8⁺ T lymphocyte induced by MVV are unresponsive to viral infection which would allow viral dissemination. Anergy and suppressor lymphocyte activity could account for this lack of CD8⁺ T lymphocyte response (McChesney and Oldstone, 1989).

The proportion of CD5⁺ cells was significantly decreased both in BALF and lung interstitium of MVV-infected animals (Watt *et al*, 1992). Reduction in the proportion of these cells indicates an ongoing process of activation in the lung during MVV-infection (Lujan *et al*, 1993), as decreased expression of ovine cell membrane CD5 follows cellular activation (Hopkins and Dutia, 1990). The role of ovine CD5⁺ T lymphocytes remains uncertain. Interestingly, in experimentally MVV-infected animals, BALF CD5⁺ T cells followed a similar trend to BALF CD4⁺ T cells. Hence, it is possible that ovine CD5⁺ T cells, as suggested for human CD5⁺ T cells (Srouf *et al*, 1980), are involved in the execution of CD4⁺ T helper functions.

During experimental MVV infection, phenotypic changes in the main T cell populations were detected as early as two weeks PI, but were maintained only for two or three weeks and then they returned to baseline levels. The short duration of the cellular immune response is of great interest, as it suggests that MVV probably temporarily eluded host defence mechanisms and experimentally infected animals

remained in an state of persistent, but latent, infection. Many animals remained in this state throughout their lives, without showing clinical signs, although a resurgence of virus replication has been observed in some animals after a quiescent period of months to years (Narayan, 1990). The outcome of MVV infection is probably influenced by many factors, namely (1) characteristics of the virus, such as virus strain, cytopathogenicity and its ability to mutate; (2) the host immune response, both humoral and cellular; and (3) genetics of the individual that may influence the susceptibility of cells to virus infection. In addition, triggering of MVV-infected macrophages by stress factors, such as concomitant pulmonary infections, could account for amplification of MVV replication, leading to development of inflammatory lung lesions and symptoms. These factors could explain the range of clinical signs and lesions that have been described in MVV-infected flocks (Watt *et al*, 1992).

During interactions between MVV-infected alveolar macrophages and lymphocytes, activation of some of the BALF T cell populations takes place. As a consequence, it is likely that several cytokines with immunoregulatory activities are simultaneously produced. These cytokines could play a major role in pathogenesis of MVV-infection, probably being the main determinant of the inflammatory nature of the viral lesions. Some of those cytokines, such as TNF- α , IL-1, IL-6 or GM-CSF, could induce accumulation of the immunocompetent cells (including lymphocytes and PMNs) in lung interstitium (potentiating lung tissue damage) and migration of these cells into the bronchoalveolar milieu. Whether or not cytokines have any indirect effects that lead to proliferation of CD8⁺ cytotoxic T cells and inhibition of CD4⁺ helper T cells is not known (Narayan *et al*, 1988b). Increased levels of cytokines in bacterial pulmonary infections have been reported. TNF- α secretion is elevated in ovine pulmonary macrophages following infection with *Corynebacterium pseudotuberculosis* and *Pasteurella haemolytica*, but not in lentivirus infection (Ellis

et al, 1991). IL-1 levels are elevated in BALF from children with bacterial pulmonary infection (Wilmott *et al*, 1990). IL-1 and TNF- α are also elevated in BALF from pigs infected with *Mycoplasma hyopneumoniae* (Asai *et al*, 1993), and IL-1 and IL-6 are elevated in the BALF of mice infected with *Chlamydia trachomatis* (Magee *et al*, 1992). In murine acquired immunodeficiency syndrome, the cytokines mainly secreted are those typical of a predominantly T_{H2} response: IL-4 and IL-10, while only small amounts of the cytokines secreted by the T_{H1} subpopulation: IL-2 and γ -IFN are found. IL-4 is the putative cytokine responsible for the induced T cell dysfunction (Kanagawa *et al*, 1993) in this syndrome. The characterisation of ovine CD4⁺ T cells into the T_{H1} and T_{H2} subtypes according to the different cytokines that they produce has not yet been performed, due to the lack of adequate reagents.

It is known that TGF- β is a very pleiotropic cytokine, which can act both as an immunosuppressive and as a potent proinflammatory molecule. The multifunctional role of this cytokine prompted the hypothesis that its dysregulation could account for some of the pulmonary pathological features of MVV infection such as: accumulation of neutrophils and T lymphocytes (Adams *et al*, 1991) in BALF and in the pulmonary interstitium of MVV-infected animals (Watt *et al*, 1992), enhanced expression of LFA-1 on BALF lymphocyte surfaces, decreased IL-2R expression by activated BALF T lymphocytes and diminished IL-2R expression on BALF lymphocytes from MVV-infected animals following mitogen stimulation. To test this hypothesis, TGF- β present in BALF supernatant and PELF was quantified during the course of experimental MVV infection. No significant differences between levels of TGF- β in BALF and PELF from control and MVV-experimentally infected animals were found. However, some participation of TGF- β in the pathogenesis of MVV infection at the lung interstitium level is still likely, as has been observed in human pulmonary fibrosis (Broekelmann *et al*, 1991). Once more it is not possible to generalise between the responses occurring within different lung compartments. It is possible that the effect of TGF- β varies in the different microenvironments or lung

compartments, and with the state of activation of the target cell (Elias *et al*, 1990). It is still possible that TGF- β , by acting in the interstitium, plays a major role in the pathogenesis of maedi.

Another cytokine with a putative multiple role in the lung is IFN. Previous *in vitro* studies (Kennedy *et al*, 1985; Narayan *et al*, 1985) have demonstrated the contribution of an ovine lentivirus-induced IFN (LV-IFN) to the pathogenesis of MVV infection (Narayan *et al*, 1985; Lairmore *et al*, 1988b). This LV-IFN has physical properties similar to IFN generated *in vitro* by other lentivirus-infected cells (Narayan *et al*, 1985). It induces MHC Class II antigen expression on alveolar macrophages (Kennedy *et al*, 1985), and as a result, the infected cell remains in a state of continuous or chronic antigen presentation. LV-IFN inhibits maturation of infected monocytes to macrophages, and thus, indirectly restricts completion of the virus life cycle. It also causes reduction in the number of transcripts of viral RNA in infected macrophages, giving rise to the phenomenon of 'slow replication'. Furthermore, as interferons inhibit the growth of fibroblasts and collagen synthesis *in vitro* (Elias *et al*, 1987), it is likely that LV-IFN regulates fibrogenesis in the lung of MVV-infected animals, where only a limited amount of fibrosis is present. The upregulated expression of MHC Class II antigens found in this work in BALF lymphocytes and macrophages suggest the active involvement of LV-IFN in the pathogenesis of MVV infection. However, our attempts to detect native ovine γ -IFN in BALF supernatant samples by using an ELISA (Emery *et al*, 1990; Rothel *et al*, 1990; Entrican *et al*, 1992) were unsuccessful. Whether these negative results were due to biological or technical reasons is not clear. In patients with sarcoidosis, attempts to quantify γ -IFN in BALF supernatants (which had been previously concentrated), by using a radioimmunoassay were also unsuccessful (Prior and Haslam, 1992). Several biological possibilities could explain the lack of detectable γ -IFN in BALF supernatant: little γ -IFN might be produced *in vivo* due to the immunosuppressive effect of pulmonary surfactant (Whilsher *et al*, 1990); locally produced γ -IFN may be

bound to receptors (Hancock *et al*, 1986) or inactivated by an inhibitor of proteases (O'Kelly *et al*, 1987); and finally, γ -IFN might be produced mainly in the interstitial lung tissue, and not pass to the alveolar spaces. The possible technical reasons to explain the lack of γ -IFN in BALF supernatant are: γ -IFN concentrations in BALF might be below the limits of the assay system employed due to the dilution occurring during BAL; and, as LV-IFN has not been fully characterised, it could be of a different type to γ -IFN which was not detected by the assay employed. LV-IFN has some characteristics of γ -IFN (Narayan *et al*, 1985; Narayan, 1986), and some properties associated with α -IFN (McInnes, 1993). Furthermore, all previous studies of the LV-IFN have been performed *in vitro*. This cytokine has not been identified *in vivo*, and its real role in the pathogenesis of maedi remains obscure.

As the lung is exposed to complex mixtures rather than to individual cytokines, and cytokines in combination have quantitatively and qualitatively different effects than they have individually (Elias *et al*, 1990; Kelley, 1990), further investigation of the interactions of each individual cytokine with other cytokines in the lung are required, although this may prove difficult.

Decreased IL-2R expression by activated BALF T lymphocytes from naturally MVV-infected animals might act as an additional contributory factor to the dysfunction of the pulmonary immune response. Whether decreased IL-2R expression is a primary factor that allows MVV replication and persistence or, on the other hand, this receptor is downregulated as a consequence of previous immunologic defects is unknown. It is however likely that the downregulation of IL-2R in BALF lymphocytes is secondary to decreased levels of IL-2 in BALF. Hence, although this study did not investigate IL-2 levels, decreased levels of IL-2 in mononuclear leukocytes culture supernatants of MVV-infected sheep have been previously reported (Ellis and DeMartini, 1985). Lower levels of IL-2 could account for decreased IL-2R expression and for a diminished T cell cytolytic function, leading to decreased immune

responsiveness in MVV-infected sheep. The finding that the different BALF T lymphocyte subpopulations of naturally MVV-infected animals did not demonstrate upregulation of IL-2R expression argues against high levels of MHC Class II antigens on BALF T lymphocytes of MVV-infected animals being solely due to the influence of cytokines. Moreover, *in vitro* studies confirmed the defective lymphocyte activation with respect to IL-2R expression observed during the natural course of the disease. The capacity of BALF T lymphocytes from MVV-infected animals to respond to exogenous mitogen stimulation was reduced, and IL-2R expression was also downregulated in unstimulated BALF T lymphocytes of MVV-infected animals when compared with those of controls.

Activation of BALF macrophages characterised by upregulation of MHC Class II molecules was also present in natural MVV infection. Coexpression of both DR and DQ MHC Class II molecules with MVV viral proteins on BALF macrophages, demonstrated by immunocytochemical techniques, has been found (Lujan *et al*, personal communication). However further studies are necessary to determine whether there is any connection between those infected BALF macrophages and the level of expression of MHC Class II molecules. As transcription and translation from the viral genome are dependent on the maturational/activational status of the cell (Gendelman *et al*, 1986), the activation of chronically infected BALF macrophages could trigger the transition from latency to active viral replication. Infection of BALF macrophages by MVV, apart from leading to the production of cytokines, might contribute to perturbation of the cellular pulmonary immune response by some other mechanisms. Cells of the macrophage lineage are the major host cell for replication of lentiviruses *in vivo* (Narayan *et al*, 1982; Peluso *et al*, 1985). If the cells involved in the non-specific cellular defence system of the host via viral antigen presentation, are at the same time the source of antigens, and are possibly damaged, this will result in disruption of the immune system and incapability of

responding efficiently to the infecting virus. Additionally, infected macrophages, by remaining in this state of continuous antigen presentation, may mediate immunopathologic disease (Narayan *et al*, 1988b).

In summary, the lung in MVV-infection acts as the major reservoir for MVV via the infection of cells from the monocyte/macrophage lineage. During natural and experimental MVV-infection the pulmonary immune response is characterised by interaction of MVV with pulmonary macrophages and lymphocytes with resultant production of cytokines. This interaction leads to a series of 'defective' immunologic phenomena. Although each of these immunologic defects individually is not sufficient to alter lung homeostasis, the combination of all of them will lead to a decline of the functional capabilities of the lung during MVV infection. Despite the presence of highly specialised effectors in the lung host, the immune surveillance mechanisms fail to control the progress of the disease, this failure leading to further immune dysfunction (Agostini *et al*, 1993). Pulmonary lesions, rather than the result of the direct action of the virus *per se*, will result from the response of diverse elements of the immune response to viral infection.

Finally, if other aspects of the biology of MVV such as antigenic drift (Narayan *et al*, 1977), sequestration of viral DNA in nuclei of infected cells (Haase *et al*, 1977) and low affinity of the induced neutralising antibodies (Kennedy-Stoskopf *et al*, 1986) are added to the failure of the host pulmonary immune response, it is clear how this lentivirus manages to escape immunological control within the lung. The current development of molecular reagents for cytokines and *in situ* techniques will probably further elucidate other aspects of the pathogenesis of this disease in the next few years.

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APPENDIX I

TABLES

Appendix I.3.1. Forward (FSC) and side (SSC) scatter parameters for BALF lymphocyte gating from control (n=13) and MVV-infected (n=12) animals. FSC values refer to the size, while SSC values refer to the complexity and internal structure of the gated cells.

CONTROLS									
<u>Sheep n°</u>	43	88	89	90	91	92	93		
FSC	69,142	60,122	50,117	48,119	60,115	53,114	51,124		
SSC	9,53	9,53	5,43	6,43	6,43	8,45	10,45		
<u>Sheep n°</u>	94	95	96	97	98	99			
FSC	57,119	44,107	55,132	67,136	36,115	59,132			
SSC	5,43	8,40	5,42	6,47	3,39	11,54			
MVV-INFECTED									
<u>Sheep n°</u>	1	2	8N	27	29	47			
FSC	48,122	59,135	60,138	58,139	52,127	57,145			
SSC	4,41	9,51	3,41	4,45	9,46	3,42			
<u>Sheep n°</u>	53	65	74	75	76	80			
FSC	43,116	50,117	55,119	58,141	62,139	49,123			
SSC	9,53	5,45	5,42	8,47	7,46	4,39			

Appendix I.3.2. Forward (FSC) and side (SSC) scatter parameters for BALF macrophage gating from control (n=13) and MVV-infected (n=12) animals. FSC values refer to the size, while SSC values refer to the complexity and internal structure of the gated cells.

<u>CONTROL</u>							
<u>Sheep n°</u>	<u>43</u>	<u>88</u>	<u>89</u>	<u>90</u>	<u>91</u>	<u>92</u>	<u>93</u>
FSC	123,255	138,255	135,255	141,255	136,255	153,255	130,255
SSC	71,255	72,255	79,255	82,255	63,255	75,255	60,255
<u>Sheep n°</u>	<u>94</u>	<u>95</u>	<u>96</u>	<u>97</u>	<u>98</u>	<u>99</u>	
FSC	133,255	121,255	145,255	113,255	149,255	67,255	
SSC	68,255	65,255	62,254	69,255	63,254	67,253	
<u>MAEDI</u>							
<u>Sheep n°</u>	<u>1</u>	<u>2</u>	<u>8N</u>	<u>27</u>	<u>29</u>	<u>47</u>	
FSC	147,255	142,255	140,255	130,255	151,255	147,255	
SSC	60,255	69,255	59,255	63,255	65,255	66,255	
<u>Sheep n°</u>	<u>53</u>	<u>65</u>	<u>74</u>	<u>75</u>	<u>76</u>	<u>80</u>	
FSC	139,255	160,255	152,255	151,255	141,255	104,254	
SSC	61,255	80,255	67,255	73,255	65,255	64,252	

Appendix I.3.3. Percentages of MHC Class II expression on BALF macrophages from control and MVV-infected animals. The MAbs employed were a pan Class II MAb (VPM36+ VPM38), VPM37 (DR specificity) and VPM41 (DQ specificity).

Sheep n°	CLASS II	VPM37	VPM41
<u>CONTROLS</u>			
43	85.08	78.34	88.53
88	93.77	12.66	91.31
89	71.97	59.46	69.96
90	90.60	23.00	79.43
91	58.36	39.67	45.28
92	87.46	66.29	78.54
93	75.94	45.75	77.66
94	45.06	22.90	38.75
95	62.24	56.13	34.89
96	76.64	43.07	67.45
97	89.16	33.13	85.01
98	48.17	41.08	22.48
99	91.06	78.06	88.51
<u>MVV-INFECTED</u>			
1	61.56	46.64	50.54
2	93.64	92.65	92.95
8N	88.42	73.53	80.32
27	82.21	75.81	80.67
29	86.68	78.01	79.67
47	70.25	68.32	79.67
53	95.13	93.93	91.53
65	82.09	71.31	70.52
74	82.09	71.31	70.52
75	91.37	89.53	90.34
76	80.81	16.45	73.43
80	81.84	20.12	78.91

Appendix I.3.4. Mean channel number of MHC Class II expression on BALF macrophages from control and MVV-infected animals. The MABs employed were a pan Class II MAb (VPM36+ VPM38), VPM37 (DR specificity) and VPM41 (DQ specificity).

Sheep n ^o	CLASS II	VPM37	VPM41
<u>CONTROLS</u>			
43	63.67	44.37	70.95
89	33.23	37.04	44.37
90	62.84	11.25	38.40
91	57.13	46.00	42.79
92	151.49	42.79	49.44
93	47.69	16.14	10.46
95	27.74	47.69	37.72
96	41.27	9.73	15.57
97	233.68	32.05	181.48
98	46.00	39.81	68.46
99	195.07	51.26	76.27
<u>MVV-INFECTED</u>			
1	41.27	33.23	46.00
2	209.69	202.25	175.04
8N	270.01	151.49	49.46
27	251.19	47.69	202.25
29	323.45	242.28	270.01
47	97.00	73.56	47.69
53	827.27	181.48	195.07
65	157.06	98.21	57.13
74	47.69	47.69	39.81
75	279.94	217.40	233.68
76	311.97	47.69	95.07

Appendix I.3.5. Mean channel number of MHC Class II expression on BALF lymphocytes from control and MVV-infected animals. The MAbs employed were a pan Class II MAb (VPM36+ VPM38), VPM37 (DR specificity) and VPM41 (DQ specificity).

Sheep n°	CLASS II	VPM37	VPM41
<u>CONTROLS</u>			
43	42.79	38.40	39.81
88	49.44	73.56	37.04
89	12.99	8.73	6.31
90	7.56	11.66	20.78
91	16.14	15.57	17.98
93	26.76	32.05	49.44
94	14.48	20.78	24.89
95	41.27	20.78	24.89
96	20.04	33.23	17.35
97	16.14	34.46	21.54
98	46.00	38.40	15.57
99	35.72	25.81	28.76
<u>MVV-INFECTED</u>			
1	15.01	16.14	14.48
2	360.46	387.47	195.07
8N	10.09	5.66	23.16
27	10.46	35.72	9.73
29	300.91	260.43	162.84
47	16.23	251.19	44.37
53	51.26	61.41	20.78
74	20.04	49.44	32.05
75	233.68	242.28	209.69
76	217.40	24.01	251.19

Appendix I.4.1. IL-2R expression by the different BALF T lymphocyte subsets of control (n=7) and MVV-infected animals (n=7). (Results expressed as the value of mean channel number of fluorescence intensity).

Sheep n ^o			
<u>CONTROL</u>	T4	T8	γδ
40	26.76	10.46	15.01
89	8.73	10.46	8.42
91	8.73	10.09	10.46
92	13.97	12.09	13.47
94	5.66	5.27	6.78
96	7.84	7.84	9.05
97	7.84	9.51	7.56
<u>MVV</u>			
17	3.81	4.09	8.85
59	7.56	8.73	8.73
79	7.84	8.73	9.05
80	17.98	16.73	17.35
99	13.47	10.85	12.09
109	2.47	4.09	8.85

Not significant (p> 0.05)

Appendix I.5.1. Forward (FSC) and side (SSC) scatter parameters for unstimulated (No Con A) and Con A-stimulated BALF lymphocyte gating from control (n=7) and MVV-infected animals (n=7). FSC refers to the size of the gated cells, while FSC indicates the complexity of the gated cells.

CONTROLS

Sheep n ^o	40	43	70	92	95	97	98	
No Con A	FSC SSC	39,143 6,57	46,149 7,71	44,158 3,54	36,147 15,56	27,150 5,63	46,149 7,71	36,138 5,56
Con A	FSC SSC	42,170 9,72	57,168 8,82	42,170 9,72	39,150 7,60	32,160 7,76	46,149 7,71	37,145 6,60

MVV-INFECTED

Sheep n ^o	20	25	28	36	68	80	97	
No Con A	FSC SSC	33,149 11,65	43,126 8,50	41,155 7,59	47,158 9,57	45,176 6,63	34,156 8,54	50,183 6,53
Con A	FSC SSC	39,165 11,78	43,126 8,77	39,218 6,85	39,208 9,86	48,198 6,76	62,149 10,43	51,225 5,78

Appendix I.5.2. Forward (FSC) and side (SSC) scatter parameters for unstimulated (No Con A) and Con A-stimulated PB lymphocyte gating from control (n=5) and MVV-infected animals (n=5). FSC refers to the size of the gated cells, while FSC indicates the complexity of the gated cells.

CONTROLS

Sheep n°		9	28	41	94	97
No Con A	FSC SSC	73,196 6,51	45,163 6,53	42,178 4,49	50,173 6,58	47,149 6,52
Con A	FSC SSC	52,248 8,95	45,178 8,58	48,244 6,70	50,233 11,98	59,226 19,131

MVV-INFECTED

Sheep n°		10	13	59	80	97
No Con A	FSC SSC	35,184 6,63	45,199 5,60	52,143 6,45	58,226 5,73	29,148 8,79
Con A	FSC SSC	29,221 12,123	40,223 6,74	58,230 12,128	58,248 8,95	33,207 11,150

Appendix I.6.1. Median and ranges of percentages of BALF lymphocytes and macrophages of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

BALF LYMPHOCYTES

Weeks PI	CON	HIC	MVV
0	6.00 (4.00-10.00)	4.50 (3.00-6.00)	9.50 (4.00-13.00)
2	16.50 (6.00-22.00)	15.50 (13.00-18.00)	28.00 (12.00-40.00)
4	6.00 (2.00-11.00)	11.00 (9.00-13.00)	36.00* (17.00-42.00)
6	14.00 (11.00-26.00)	12.00 (11.00-13.00)	28.50 (10.00-54.00)
8	13.50 (13.00-18.00)	15.00 (15.00-15.00)	26.00 (12.00-52.00)
12	15.50 (13.00-16.00)		23.00 (8.00-40.00)
16	9.50 (10.00-18.00)		17.00 (10.00-26.00)

BALF MACROPHAGES

Weeks PI	CON	HIC	MVV
0	89.00 (86.00-93.00)	88.50 (82.00-95.00)	82.00 (76.00-91.00)
2	79.00 (77.00-80.00)	81.00 (81.00-81.00)	70.00 (58.00-86.00)
4	93.00 (87.00-97.00)	85.00 (80.00-90.00)	61.00 (56.00-82.00)
6	83.50 (68.00-88.00)	83.50 (80.00-87.00)	64.00 (42.00-87.00)
8	78.50 (59.00-82.00)	84.00 (84.00-84.00)	72.00 (40.00-85.00)
12	81.50 (55.00-86.00)		75.00 (56.00-86.00)
16	82.50 (81.00-85.00)		79.00 (70.00-84.00)

* Significantly different from other two groups at $p < 0.05$ (Kruskal-Wallis test).

Appendix I.6.2. Median and ranges of percentages of BALF neutrophils of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

BALF NEUTROPHILS			
Weeks PI	CON	HIC	MVV
0	2.50 (1.00-3.00)	5.50 (1.00-10.00)	7.50 (1.00-10.00)
2	2.50-(1.00-5.00)	3.00 (1.00-5.00)	2.00 (1.00-3.00)
4	2.00 (1.00-2.00)	4.00 (1.00-7.00)	1.50 (1.00-4.00)
6	2.50 (1.00-6.00)	4.00 (2.00-6.00)	3.50 (1.00-14.00)
8	7.00 (2.00-27.00)	1.00 (1.00-1.00)	2.00 (0.00-8.00)
12	1.50 (1.00-3.00)		3.50 (1.00-6.00)
16	3.00 (1.00-5.00)		3.00 (1.00-6.00)

Appendix I.6.3 Median and ranges of percentages of BALF CD4⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	44.28 (36.26-58.20)	45.18 (32.20-58.20)	44.49 (38.86-56.47)
2	41.46 (37.82-55.77)	51.92 (38.70-65.20)	40.26 (31.98-60.22)
4	38.30 (31.89-64.92)	51.83 (31.90-71.80)	56.92 (27.26-38.76)
6	51.24 (41.94-58.55)	56.92 (45.80-68.10)	30.76* (12.85-44.48)
8	45.86 (44.36-63.66)	58.78 (58.78-58.78)	28.09* (8.11-42.46)
12	50.59 (35.29-58.04)		33.19* (17.62-39.62)
16	44.70 (29.90-60.20)		33.94 (16.71-60.52)

Appendix I.6.4. Median and ranges of percentages of BALF CD8⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	19.49 (13.48-27.31)	17.02 (13.08-20.97)	18.89 (9.58-27.69)
2	19.22 (12.92-33.93)	15.86 (11.03-20.70)	33.61* (22.96-54.75)
4	13.19 (11.97-17.21)	13.40 (12.79-14.01)	59.33* (30.34-63.55)
6	18.10 (17.17-25.88)	15.14 (11.42-18.85)	52.26* (20.25-68.76)
8	19.92 (17.89-27.67)	14.55 (10.98-20.56)	55.33* (19.45-70.60)
12	17.87 (11.04-31.76)		35.65 (15.75-62.95)
16	15.98 (13.98-17.99)		39.98 (12.26-73.05)

* Significantly different from other two groups at p< 0.05 (Kruskal-Wallis test).

Appendix I.6.5. Median and ranges of percentages of BALF CD5⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	46.83 (35.84-59.20)	44.20 (29.40-59.00)	43.70 (32.25-59.55)
2	44.69 (40.00-49.77)	51.71 (42.05-61.37)	45.36 (24.70-62.32)
4	46.39 (27.90-60.17)	54.39 (34.70-74.10)	30.01 (23.37-39.01)
6	54.04 (42.74-60.15)	56.98 (42.50-71.50)	27.55* (12.46-46.48)
8	45.57 (42.07-59.96)	52.00 (52.00-52.00)	28.95* (14.73-43.53)
12	49.21 (33.26-58.87)		34.39 (21.21-36.65)
16	43.02 (32.30-53.70)		32.35 (16.22-58.25)

Appendix I.6.6. Median and ranges of percentages of BALF $\gamma\delta^+$ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	9.06 (5.02-14.71)	8.22 (4.65-11.79)	6.82 (5.53-11.86)
2	9.02 (9.00-14.37)	11.17 (10.75-11.59)	5.10 (2.28-6.98)
4	9.74 (4.14-15.44)	8.68 (8.42-8.94)	4.66 (1.68-8.69)
6	12.54 (9.52-17.41)	10.15 (5.50-10.80)	3.16* (1.61-8.29)
8	12.42 (10.12-17.51)	11.35 (11.35-11.35)	5.49* (2.83-7.71)
12	12.03 (5.95-14.20)		6.12 (3.04-8.82)
16	12.93 (8.95-15.92)		8.97 (3.08-10.94)

* Significantly different from other two groups at p<0.05 (Kruskal-Wallis test).

Appendix I.6.7. Median and ranges of BALF CD4⁺/CD8⁺ ratio of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). **PI**= post-inoculation.

Weeks PI	CON	HIC	MVV
0	2.25 (1.52-4.31)	2.98 (1.53-4.44)	2.45 (1.60-5.89)
2	2.47 (1.14-3.16)	3.88 (1.86-5.90)	1.17 (0.58-2.62)
4	2.97 (2.51-3.77)	3.92 (2.27-5.57)	0.47* (0.44-1.27)
6	2.72 (1.92-3.14)	4.19 (2.42-5.96)	0.59* (0.25-2.19)
8	2.50 (1.80-2.96)	4.03 (4.03-4.03)	0.50* (0.13-2.15)
12	3.10 (1.52-3.19)		1.03* (0.30-2.36)
16	2.96 (1.62-4.30)		1.23 (0.22-12.28)

Appendix I.6.8. Median and ranges of percentages of BALF CD4⁺/VPM54⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). **PI**= post-inoculation.

Weeks PI	CON	HIC	MVV
0	3.61 (1.36-7.30)	5.76 (5.07-6.45)	4.20 (1.30-8.73)
2	14.14 (4.03-30.19)	6.58 (5.39-7.78)	19.39 (10.13-32.69)
4	15.73 (6.73-20.40)	9.78 (6.76-12.81)	14.60 (7.41-24.06)
6	20.44 (14.70-36.30)	19.33 (17.97-20.68)	14.44 (8.28-20.71)
8	29.48 (18.58-41.51)	11.01 (11.01-11.01)	21.37 (11.09-53.74)
12	34.93 (23.32-43.53)		29.17 (18.30-39.25)
16	25.00 (17.25-32.75)		23.25 (16.82-38.72)

* Significantly different from other two groups at p<0.05 (Kruskal-Wallis test).

Appendix I.6.9. Median and ranges of percentages of BALF CD4⁺/VPM36⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	1.76 (1.05-5.12)	3.05 (2.69-3.42)	3.03 (1.25-8.85)
2	6.18 (2.83-11.64)	2.97 (2.63-3.31)	17.91* (8.84-34.98)
4	10.28 (4.40-15.33)	9.66 (9.24-10.09)	11.44 (4.00-23.35)
6	16.06 (6.78-18.78)	11.44 (9.51-13.38)	11.17 (6.91-16.16)
8	27.00 (11.55-41.07)	15.26 (15.26-15.26)	16.53 (8.36-56.96)
12	23.69 (19.40-29.44)		21.98 (15.19-27.84)
16	10.65 (9.65-11.64)		16.50 (9.51-53.21)

Appendix I.6.10. Median and ranges of percentages of BALF CD4⁺/IL-2R⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	10.76 (8.75-16.53)	16.24 (7.13-25.35)	16.06 (9.79-20.77)
2	14.50 (6.31-25.34)	21.55 (16.74-26.36)	32.98 (17.34-39.58)
4	23.45 (17.29-53.88)	37.22 (28.92-45.52)	20.02 (5.92-34.74)
6	36.05 (32.93-46.64)	54.42* (52.53-56.32)	20.26 (10.13-30.74)
8	42.14 (35.89-44.43)	44.83 (44.83-44.83)	30.83 (15.86-47.74)
12	54.78 (36.95-66.69)		30.18 (18.60-50.49)
16	45.11 (37.26-52.97)		23.81 (12.57-38.33)

* Significantly different from other two groups at p< 0.05 (Kruskal-Wallis test).

Appendix I.6.11. Median and ranges of percentages of BALF CD8⁺/VPM54⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). **PI**= post-inoculation.

Weeks PI	CON	HIC	MVV
0	5.87 (5.13-14.60)	13.14 (8.50-17.78)	10.97 (9.28-21.57)
2	14.56 (6.75-32.59)	10.85 (9.04-12.66)	22.05 (5.51-33.40)
4	29.33 (13.70-40.93)	23.39 (16.48-30.30)	20.81 (13.93-40.51)
6	34.47 (38.13-52.42)	20.69 (16.95-24.43)	29.50 (14.56-55.17)
8	43.17 (34.87-62.96)	18.80 (18.80-18.80)	30.95 (6.00-81.50)
12	38.10 (36.20-50.18)		39.03 (23.93-55.62)
16	31.44 (26.06-36.82)		48.17 (36.07-56.52)

Appendix I.6.12. Median and ranges of percentages of BALF CD8⁺/VPM36⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). **PI**= post-inoculation.

Weeks PI	CON	HIC	MVV
0	2.52 (1.14-7.69)	3.48 (1.90-5.07)	6.19 (2.80-17.53)
2	13.13 (2.68-19.46)	7.12 (5.62-8.63)	14.97 (3.18-42.56)
4	16.16 (6.99-41.82)	23.85 (20.60-27.10)	6.31 (5.00-18.12)
6	27.93 (16.98-28.38)	13.05 (12.40-13.70)	13.48 (7.70-50.49)
8	38.03 (17.60-64.70)	14.50 (14.50-14.50)	17.51 (1.99-49.01)
12	33.43 (27.33-46.21)		26.39 (13.27-32.51)
16	17.85 (13.98-21.72)		25.23 (15.00-49.52)

Appendix I.6.13. Median and ranges of percentages of BALF CD8⁺/IL-2R⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	1.83 (1.31-4.94)	12.86 (1.10-24.60)	5.85 (1.58-8.02)
2	4.84 (1.10-8.15)	5.27 (4.83-5.71)	7.35 (1.67-10.50)
4	16.19 (3.07-32.31)	18.40 (14.89-21.92)	6.28 (3.72-10.58)
6	14.31 (9.13-18.89)	13.54 (9.71-17.37)	7.16 (1.54-51.70)
8	16.13 (13.25-29.15)	16.08 (16.08-16.08)	15.56 (4.19-21.41)
12	20.03 (14.90-28.13)		10.01 (6.91-28.93)
16	22.74 (22.22-23.27)		9.41 (6.40-44.11)

Appendix I.6.14. Median and ranges of percentages of BALF $\gamma\delta^+$ /VPM54⁺ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	1.96 (1.15-3.90)	4.31 (1.93-6.70)	8.04 (1.17-10.52)
2	15.05 (5.46-18.10)	7.01 (4.48-9.55)	12.58 (10.34-33.33)
4	9.50 (7.99-12.35)	16.51 (14.86-18.16)	31.76 (12.59-59.67)
6	13.70 (12.18-18.94)	10.78 (10.00-11.56)	22.92 (11.73-32.65)
8	27.40 (16.75-37.20)	13.16 (13.16-13.16)	33.19 (19.18-60.84)
12	24.86 (22.25-49.32)		28.98 (13.07-38.25)
16	25.00 (21.62-24.26)		30.02 (22.66-43.92)

Appendix I.6.15. Median and ranges of percentages of BALF $\gamma\delta^+$ /VPM36 $^+$ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	1.88 (1.26-2.36)	4.38 (2.75-6.01)	6.61 (0.19-8.43)
2	9.56 (4.27-20.62)	9.19 (5.71-12.68)	10.30 (8.62-31.01)
4	9.14 (5.37-35.25)	10.73 (10.29-11.18)	23.62 (12.90-34.45)
6	12.06 (9.28-15.99)	11.08 (7.51-14.65)	12.18 (5.57-34.24)
8	21.23 (1.69-35.45)	12.79 (12.79-12.79)	24.47 (13.51-62.89)
12	19.88 (13.93-25.08)		19.41 (11.49-37.04)
16	13.64 (9.45-17.82)		17.73 (10.22-56.75)

Appendix I.6.16. Median and ranges of percentages of BALF $\gamma\delta^+$ /IL-2R $^+$ T cells of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). PI= post-inoculation.

Weeks PI	CON	HIC	MVV
0	20.50 (10.23-42.41)	35.38 (22.70-48.00)	25.01 (14.21-48.60)
2	32.28 (12.05-36.19)	44.76 (23.90-65.70)	42.68 (25.66-67.50)
4	37.56 (25.30-74.10)	64.88 (55.73-74.03)	52.05 (37.30-70.55)
6	65.33 (57.27-75.26)	55.19 (54.08-56.29)	60.01 (48.19-64.25)
8	62.03 (45.91-81.98)	74.72 (74.72-56.29)	46.82 (37.65-76.09)
12	72.22 (31.00-90.30)		52.51 (37.09-63.04)
16	65.42 (50.60-80.30)		42.16 (16.93-72.42)

Appendix I.6.17. Urea concentrations of plasma and BALF (mg/dl) of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6).

Sheep n ^o	Week [*]	PLASMA	BALF
CON 70	2	24.70	0.84
"	4	28.31	0.39
"	6	46.38	0.68
"	8	34.93	0.59
"	12	30.12	0.34
"	16	27.10	0.89
CON 107	2	24.09	0.19
"	4	43.37	0.34
"	6	54.21	0.45
"	8	44.57	0.48
"	12	30.72	0.46
"	16	35.54	0.25
CON 125	2	33.13	1.11
"	4	27.10	1.28
"	6	42.77	1.16
"	8	30.12	0.49
"	12	36.14	0.49
CON 129	2	34.33	1.06
"	4	28.91	0.68
"	6	34.93	0.74
"	8	22.28	0.41
"	12	33.13	0.42
HIC 141	2	43.97	0.98
"	4	43.37	0.86
"	6	37.95	0.59
"	8	27.71	0.62
HIC 35	2	37.95	1.10
"	4	48.79	0.85
"	6	38.55	1.07

* Weeks post-inoculation.

Appendix I.6.17. (Continued).

Sheep n^o	Week[*]	PLASMA	BALF
MVV 14	2	36.74	0.38
"	4	28.31	0.28
"	6	33.13	0.61
"	8	40.36	0.52
"	12	26.50	0.60
"	16	25.30	0.20
MVV 56	2	45.18	0.24
"	4	38.55	0.93
"	6	36.74	0.02
"	8	32.89	0.50
"	12	19.87	0.07
"	16	39.15	0.37
MVV 95	2	42.16	0.74
"	4	34.93	0.46
"	6	31.92	0.57
"	8	33.73	0.61
"	12	13.25	0.63
"	16	33.73	0.97
MVV 102	2	40.36	0.69
"	4	43.37	0.46
"	6	38.55	0.45
"	8	37.34	0.49
"	12	15.66	0.59
"	16	45.18	0.54
MVV 105	2	31.32	0.66
"	4	34.33	0.58
"	6	38.55	0.72
"	8	30.72	0.73
"	12	21.08	0.83
"	16	39.15	0.94
MVV 120	2	31.92	0.52
"	4	45.18	0.64
"	6	40.36	0.61
"	8	38.55	0.73
"	12	23.49	0.55
"	16	38.55	0.95

* Weeks post-inoculation.

Appendix I.6.18. TGF- β concentrations in plasma (ng/ml) of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6).

Week *	Sheep n ^o	TGF- β	Sheep n ^o	TGF- β
2	CON 70	5.05	MVV 14	0.44
4	"	8.87	"	5.90
6	"	13.90	"	10.71
8	"	10.40	"	7.18
12	"	11.86	"	5.95
16	"	9.64	"	10.40
2	CON 107	0.44	MVV 56	12.02
4	"	3.83	"	17.78
6	"	11.64	"	8.39
8	"	7.74	"	9.10
12	"	16.45	"	7.11
16	"	11.35	"	16.60
2	CON 125	15.31	MVV 95	6.02
4	"	17.78	"	9.18
6	"	20.99	"	6.54
8	"	19.91	"	9.89
12	"	29.66	"	7.53
16			"	15.10
2	CON 129	5.61	MVV 102	3.77
4	"	2.70	"	3.26
6	"	6.09	"	6.20
8	"	4.84	"	3.32
12	"	7.08	"	5.50
16			"	9.64
2	HIC 35	12.56	MVV 105	12.79
4	"	8.32	"	15.81
6	"	14.32	"	19.91
8			"	14.69
12			"	17.34
16			"	8.11
2	HIC 141	9.33	MVV 120	15.81
4	"	11.80	"	6.87
6	"	6.65	"	7.51
8	"	7.03	"	7.85
12			"	5.22
16			"	16.60

* Weeks post-inoculation.

Appendix I.6.19. TGF- β concentrations in BALF (ng/ml) of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6).

Week*	Sheep n°	TGF- β	Sheep n°	TGF- β
2	CON 70	13.99	MVV 14	22.43
4	"	12.43	"	29.46
6	"	11.33	"	23.95
8	"	24.22	"	25.96
12	"	14.59	"	21.44
16	"	28.96	"	26.26
2	CON 107	15.64	MVV 56	16.57
4	"	25.96	"	20.86
6	"	19.73	"	28.14
8	"	48.90	"	17.35
12	"	22.61	"	24.22
16	"	35.02	"	32.68
2	CON 125	13.62	MVV 95	98.46
4	"	18.16	"	30.50
6	"	17.35	"	83.51
8	"	18.98	"	72.33
12	"	76.61	"	36.67
16			"	26.66
2	CON 129	13.59	MVV 102	15.11
4	"	11.12	"	10.76
6	"	17.55	"	13.97
8	"	13.51	"	15.00
12	"	38.84	"	13.99
16			"	12.14
2	HIC 35	32.68	MVV 105	22.61
4	"	18.46	"	68.90
6	"	16.17	"	16.76
8			"	57.80
12			"	21.84
16			"	59.47
2	HIC 141	16.19	MVV 120	19.24
4	"	15.46	"	34.22
6	"	16.00	"	22.09
8	"	13.94	"	65.21
12			"	50.04
16			"	50.04

* Weeks post-inoculation.

Appendix I.6.20. TGF- β concentrations in PELF (ng/ml) of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6).

Week *	Sheep n ^o	TGF- β	Sheep n ^o	TGF- β
2	CON 70	412.28	MVV 14	2204.42
4	"	909.87	"	2940.69
6	"	796.08	"	1309.50
8	"	1426.80	"	2031.88
12	"	1310.91	"	958.15
16	"	882.41	"	3435.85
2	CON 107	2087.94	MVV 56	3226.17
4	"	3349.61	"	866.31
6	"	2370.55	"	3820.00
8	"	4569.70	"	2515.22
12	"	1496.55	"	622.45
16	"	5735.92	"	3544.79
2	CON 125	255.37	MVV 95	5650.00
4	"	396.15	"	2296.04
6	"	639.34	"	4700.00
8	"	1166.89	"	4026.61
12	"	5652.28	"	768.96
16	"		"	895.02
2	CON 129	441.40	MVV 102	892.54
4	"	470.48	"	1005.52
6	"	834.32	"	1193.59
8	"	729.81	"	1143.45
12	"	3023.30	"	369.47
16	"		"	1012.96
2	HIC 35	1131.70	MVV 105	1077.14
4	"	1058.49	"	3989.85
6	"	582.44	"	982.80
8			"	2463.00
12			"	558.01
16			"	2473.95
2	HIC 141	726.60	MVV 120	1162.09
4	"	776.24	"	2399.18
6	"	1020.48	"	1920.94
8	"	624.43	"	3420.19
12			"	2120.19
16			"	2022.11

* Weeks post-inoculation.

Appendix I.6.21. Median and ranges of TGF- β concentrations in plasma (ng/ml) of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). **PI**= post-inoculation.

Weeks PI	CON	HIC	MVV
2	5.33 (0.44-15.31)	10.94 (9.33-12.56)	9.02 (0.44-15.81)
4	6.35 (2.70-17.78)	10.06 (8.32-11.80)	8.02 (3.26-17.78)
6	12.77 (6.09-20.99)	10.50 (6.65-14.32)	7.95 (6.20-19.91)
8	9.07 (4.84-19.91)	7.03 (7.03- 7.03)	8.47 (3.32-14.69)
12	14.16 (7.08-29.66)		6.53 (5.22-17.34)
16	10.50 (9.64-11.35)		15.10 (8.11-16.60)

Appendix I.6.22. Median and ranges of TGF- β concentrations in PELF (ng/ml) of animals employed for experimental MVV infection at different sampling times. Control sheep (CON) (n=4), sheep inoculated with heat inactivated MVV (HIC) (n=2) and MVV-inoculated sheep (MVV) (n=6). **PI**= post-inoculation.

Weeks PI	CON	HIC	MVV
2	427 (255-2088)	929 (727-1132)	1683 (893-5650)
4	690 (396-3350)	917 (776-1058)	2348 (866-3990)
6	802 (639-2371)	802 (582-1020)	1615 (983-4700)
8	1297 (730-4570)	624 (624-624)	2489 (1143-4027)
12	2260 (1311-5652)		696 (369-2120)
16	3309 (882-5736)		2248 (895-3545)

APPENDIX II

BUFFERS AND REAGENTS

1. Immunofluorescence Buffer (PBA)

Bovine serum albumin (BSA) (Sigma, Poole, UK)	1%
Sodium azide (Sigma, Poole, UK)	0.1%
Heparin (Leo Laboratories, Princes Risborough, UK)	20 IU/ml

Make up in Phosphate Buffered Saline (PBS) pH 7.2 (see Appendix I.2).

2. Paraformaldehyde Fixative

(a) Preparation of PBS pH 7.2

Sodium Chloride (NaCl)	8.00 g/l
Potassium Chloride (KCl)	0.20 g/l
Disodium hydrogen phosphate (Na_2HPO_4)	1.15 g/l
Potassium dihydrogen phosphate (KH_2PO_4)	0.20 g/l

Dissolve in 1 l distilled water

(b) Dissolve 1 g paraformaldehyde in 50 ml warm PBS. Add 1M sodium hydroxide dropwise until the paraformaldehyde dissolves. Adjust pH to 7.2 and store at 4°C.

3. Tris-ammonium Chloride Buffer ($\text{Tris-NH}_4\text{Cl}$)

(a) 0.17M Tris (hydroxymethyl) aminomethane	20.6 g
Distilled water	1000 ml

(b) 0.16M Ammonium Chloride 8.3 g
Distilled water 1000 ml

(c) To prepare lysing solution add 10 ml of (a) to 90 ml of (b) and adjust pH to 7.2 using 1M HCl.

4. RPMI 1640 Supplemented Culture Medium

RPMI 1640 (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine, 100 U/ml benzylpenicillin, 100 U/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol, 2 g/l sodium bicarbonate and 10 % inactivated foetal calf serum (FCS) (Ato Tek, Hereford, UK).

5. RPMI 1640 Complete Culture Medium

This medium was prepared as the RPMI 1640 "Supplemented Culture Medium" but also contained 5000 U/ml mycostatin (Nystatin, Squibb, Upjohn, Crawley, UK) and 100 U/ml gentamicin.

6. RPMI 1640 Wash Culture Medium

RPMI 1640 (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine, 100 U/ml benzyl penicillin, 100 U/ml streptomycin, 2 g/l sodium bicarbonate and 1% FCS.

7. Supplemented Dulbecco's modified Eagle medium (DMEM)

Dulbecco's modified Eagle medium (DMEM) (Gibco, Paisley, Scotland) supplemented with 10% heat-inactivated FCS.

8. 0.02% Versene

To make 5 l:

(a) Dissolve 1 g EDTA disodium salt (BDH, Poole, UK) in 2 l of PBS.

(b) Adjust pH to 7.1-7.3.

(c) Make up to 5 l with PBS.

(d) Dispense into aliquots as required.

(e) Autoclave at 1516 g for 2 min.

9. Trypsin/Versene

(a) 1 volume of 0.25% trypsin (DIFCO, Detroit, Michigan, USA).

(b) 4 volumes of 0.02% versene.

APPENDIX III

PROTOCOLS

1. Lymphoprep (Density gradient) Separation of Peripheral Blood Leucocytes.

(a) Collect 20 ml of jugular blood into sterile glass universals containing 4-5 mm sterile glass beads.

(b) Defibrinate the blood by vigorous shaking for 5-10 min or until a discernible white fibrin clot forms.

(c) After allowing the clot to settle down, pipette off the defibrinated blood from the top.

(d) Gently layer the defibrinated blood over 10 ml of Lymphoprep (Nycomed, Birmingham, UK) and centrifuge at 800 g for 20 min at 4°C, with the brake off to prevent excessive deceleration.

(e) Using a sterile long tipped Pasteur pipette recover the lymphocytes and monocytes from the Lymphoprep/plasma interphase.

(f) Wash cells thoroughly for three times in RPMI 1640 "culture wash medium" (Appendix II.6) by centrifugation at 350 g for 10 min at 4°C.

(g) Resuspend cells at 2×10^5 /ml in RPMI 1640 "supplemented culture medium" (Appendix II.4).

2. Separation of PBL by Hypotonic Lysis with Tris-ammonium Chloride

- (a) Prepare Tris-ammonium chloride as detailed in Appendix II.3.
- (b) Collect 10 ml jugular blood into heparinised vacutainer (Becton-Dickinson, Rutherford, USA).
- (b) Mix blood with 35 ml 37°C tris-ammonium chloride and allow to stand at room temperature for approximately 3 min for complete lysis of the red blood cells to occur.
- (c) Centrifuge at 800 g for 15 min at 4°C and discard supernatant.
- (d) Resuspend cell pellet, wash three times, count and adjust cell concentration as above.

3. Separation of Peripheral Blood Leucocytes by Osmotic Shock Lysis

- (a) Collect 10 ml jugular blood into heparinised vacutainer (Becton-Dickinson, Rutherford, USA).
- (b) Centrifuge the samples at 800 g for 10 min.
- (c) Collect the leucocyte rich supernatant and resuspend it in 10 ml PBS.
- (d) Add 20 ml double deionised distilled water and mix quickly.
- (e) After 30 s, add 2ml 10 x PBS to restore the isotonicity.
- (f) Centrifuge the samples at 800 g for 5 min.

(g) Discard the supernatant and resuspend the cell pellet in 5 ml PBS.

(h) Wash three times in PBA (Appendix II.1)

4. Toluidine Blue Staining Technique

Cytospin preparations, prepared and fixed as described previously (Chapter 2), were stained overnight in 0.5% toluidine blue (Sigma, Poole, UK) in 0.7N HCl at room temperature, washed quickly in distilled water, air dried and mounted in DPX neutral medium (BHD, Poole, UK).

5. Leishman's Staining Technique

Cytospin preparations, prepared and fixed as described previously (Chapter 2), were covered with one volume of Leishman's staining solution, (BDH, Poole, UK) and incubated at room temperature for 2 min. Two volumes of Leishman's buffer (pH 6.8) were then added to completely cover the slide and left for 8 min. The preparations were finally washed in Leishman's buffer (pH 6.8), blotted dry and mounted in DPX neutral medium.

6. Trypan Blue Exclusion

Trypan blue is one of the several stains recommended for use in dye exclusion procedures for viable cell counting (Hunt, 1987). This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do.

(a) In an Eppendorf tube, 20 μ l of the BALF cell suspension are mixed with 20 μ l of a 0.4% Trypan blue solution (Sigma, Poole, UK) and resuspended with a pipette tip.

(b) Without delay, 12 μl of the previous mixture are run into a haemocytometer chamber (Improved Neubauer, Hawksley and Sons, London) and 1 min is allowed for the cells to settle.

(c) Within the next 5 min, blue cells (dead) versus unstained cells (live) are scored, counting all the cells in the four 1 mm corner squares of the haemocytometer chamber and keeping a separate count of viable and non-viable cells. Erythrocytes are ignored.

(d) Cell counts were determined following the following calculations:

- Cells per ml: the average count per square \times dilution factor $\times 10^4$.

- Total Cells: cells per ml \times the original volume of fluid from which cell sample was removed.

- % Cell Viability: total viable cells (unstained)/ total cells (stained + unstained) $\times 100$.

APPENDIX IV

PUBLISHED PAPERS

Phenotypic analysis of cells in bronchoalveolar lavage fluid and peripheral blood of maedi visna-infected sheep

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(Accepted for publication 23 September 1992)

SUMMARY

A phenotypic analysis of bronchoalveolar lavage fluid (BALF) and peripheral blood (PB) cells in maedi visna virus (MVV)-infected sheep has been performed. The differential cell count in BALF from MVV-infected animals was characterized by a significant increase ($P < 0.05$) in lymphocytes and neutrophils. Lymphocyte phenotyping in BALF from MVV-infected sheep showed a significant decrease ($P < 0.05$) of CD4⁺ cells, a significant increase ($P < 0.05$) of CD8⁺ cells and a significant inversion ($P < 0.001$) of the CD4⁺/CD8⁺ ratio. CD5⁺ lymphocytes were also significantly decreased ($P < 0.05$). $\gamma\delta$ T cells and B cells did not differ significantly when compared with the controls. No correlation was observed between BALF and PB lymphocyte phenotypes. BALF macrophages from MVV-infected animals showed increased MHC class II expression and BALF lymphocytes from the same animals demonstrated up-regulation of LFA-1 and LFA-3 expression. These findings and their relationship with lentiviral pathogenesis are discussed.

Keywords maedi lentivirus bronchoalveolar lavage cell phenotype sheep

INTRODUCTION

Maedi visna virus (MVV) is a lentivirus responsible for an ovine multisystemic disease, a chronic progressive interstitial pneumonia being one of its main clinical manifestations [1,2]. Other manifestations are meningoencephalitis, indurative mastitis and arthritis [3-5]. The virus causes persistent infection of the monocyte/macrophage cell lineage leading to chronic inflammatory lesions [6,7]. In the interstitial lung disease, lesions consist of interstitial mononuclear cell infiltration, lymphoid follicle and smooth muscle hyperplasia [8]. The study of the interstitial lung diseases both in humans and animals is a vast research field, the etiology of most of these diseases still being unknown. The study of the immune response in the lungs during the course of MVV infection might provide useful information about the pathogenesis of the lentiviral-induced interstitial pneumonias, including the lymphocytic pneumonia observed in DS [9]. This study reports the phenotypic alterations induced by natural MVV infection in cells recovered by bronchoalveolar lavage (BAL) and the absence of changes in peripheral blood lymphocytes (PBL).

MATERIAL AND METHODS

Animals, bronchoalveolar lavage technique and sample processing

Twelve adult Texel ewes, seropositive for MVV by the agar gel immunodiffusion test (AGID) [10] and 13 breed, sex, and age-matched seronegative control ewes were used. The seropositive animals were selected from a naturally infected flock [11] and represented different stages of clinical respiratory disease severity, as assessed by pulmonary function testing (Collie *et al.*, *Am J Vet Res*, manuscript accepted). The control animals were purchased from an accredited MVV-free Texel flock. Animals were kept in separate buildings but under similar management conditions.

Animals were anaesthetized with intravenous thiopentone sodium (20 mg/kg) (Intraval Sodium; May & Baker Ltd), intubated with 9.5-10.5 mm cuffed endotracheal tubes and placed in sternal recumbency. A fiberoptic bronchoscope (American Optical) was introduced through the endotracheal tube and wedged in the lobar bronchus of the diaphragmatic lobe. Two hundred millilitres of sterile saline solution, divided into four aliquots of 50 ml, were infused through the lumen of the bronchoscope. A gentle aspiration of the bronchoalveolar lavage fluid (BALF) was carried out after each of the infusions and the four recoveries were pooled together in precooled plastic containers. Peripheral blood (PB) samples were taken from each animal by jugular venepuncture and collected into EDTA tubes.

BALF was filtered through a loose cotton gauze and the recovered volume measured, the normal recovery being between

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40% and 60%. Cell pellets were harvested by a 10 min centrifugation at 800 *g* and resuspended in sterile PBS. A total BALF cell count was performed by using an automatic counter (Serono, Baker Diagnostics). Viability of the cells, determined by trypan blue dye exclusion, was always higher than 90%. Finally, cytocentrifuge smears were prepared (Cytospin 3, Shandon) and stained with Leishmann's dye for differential cell counting. In blood, erythrocytes were lysed with NH_4Cl buffer [12] and a total and differential leucocyte count was performed.

Immunofluorescent labelling technique

The reactivity of the MoAbs used in this study was: Leucocyte Common Antigen (LCA, CD45 analogue) (VPM 18), CD4 (17D.13), CD8 (SBU-T8), $\gamma\delta$ TCR (86D), CD5 (ST1), IgG light chain for B cells (VPM 8), a Pan MHC class II reagent (VPM 36+VPM 38), an MHC class II-DR (VPM 37), an MHC class II-DQ (VPM 41) and the Lymphocyte Function-Associated Antigen-1 (LFA-1) (F10-150-39) and 3 (LFA-3) (L180/1) [13-21]. All MoAbs were used on lymphocytes and macrophages recovered from BALF, while only the first seven MoAbs were used for analysis of lymphocytes in PB. MoAbs were used as hybridoma supernatant fluids at previously determined optimal dilutions.

The procedure was essentially the same for both BALF and PB samples. Cells (1.5×10^5) were incubated with 50 μl of primary MoAbs for 45 min at 4°C. Cells were then washed three times with PBA (PBS+2% bovine serum albumin +0.1% sodium azide) and incubated with 50 μl of a 1:100 dilution FITC-conjugated rabbit F(ab')₂ anti-mouse immunoglobulin (whole molecule) (Dako) for 30 min at 4°C in the dark. Three further washes with PBA were performed and cells were finally fixed in a 1% paraformaldehyde solution (pH 7.2) and kept at 4°C until analysed within 24 h.

Flow cytometry, data analysis and statistics

Samples were analysed on a FACScan flow cytometer (Becton Dickinson). Ten thousand individual cells from each sample were analysed by setting separate live gates around the lymphocyte or macrophage populations respectively, being distinguished by their FSC/SSC profiles. The macrophage autofluorescence was corrected by setting an appropriate fluorescence threshold value for each animal by using control cells stained without the addition of MoAb. Consort 30 and Lysis software packages were used to analyse data. The results were expressed either as the percentage of cells positively stained or as the value of the mode channel number of fluorescence intensity. All the sample comparisons between MVV-infected

and control animals were made using the Mann-Whitney non-parametric rank test.

RESULTS

Total and differential BALF cell count

BALF from MVV-infected animals presented a slightly increased cellularity when compared with controls (MVV ($n=12$) = 3.9×10^6 (range 0.2-6.3); control ($n=13$) = 2.5×10^6 (range 1.6-6.9) (values expressed as median of cells per millilitre)) although this difference was not significant. MVV-infected animals also had significantly increased ($P < 0.05$) lymphocytes and neutrophils and significantly decreased macrophages ($P < 0.05$) (Table 1). However, absolute number showed a significant increase of lymphocytes ($P < 0.001$), neutrophils ($P < 0.001$) and macrophages ($P < 0.05$) (data not shown).

Lymphocyte phenotyping in BALF and PB (Table 2)

In BALF of MVV-infected animals, the most noticeable changes were a significant decrease ($P < 0.05$) of CD4⁺ lymphocytes and a significant increase ($P < 0.05$) of CD8⁺ T lymphocytes ratios, leading to a significant inversion ($P < 0.001$) of the CD4⁺/CD8⁺ ratio when compared with controls. A significant decrease ($P < 0.05$) of CD5⁺ lymphocytes was also observed. The percentage of $\gamma\delta$ T cells in BALF did not show statistical variations and B cells in BALF of MVV-infected animals were increased, although this difference was not statistically significant. In PB, no significant differences in lymphocyte subsets between control and MVV-infected sheep were found for any of the reagents used (data not shown). The CD4⁺/CD8⁺ ratio in

Table 1. Differential cell count in bronchoalveolar lavage fluid (BALF) from control and maedi visna virus (MVV)-infected animals

	Control ($n=9$)	Maedi ($n=12$)
Neutrophils	1.0 (0.0-3.0)*	5.5† (1.0-30.0)
Lymphocytes	5.0 (3.0-20.0)	13.0† (6.0-57.0)
Macrophages	86.0 (79.0-91.0)	69.5† (37.0-91.0)
Eosinophils	4.0 (0.0-11.0)	1.0 (0.0-9.0)
Mast cells	1.0 (0.0-2.0)	0.5 (0.0-2.0)

* Results are expressed as median of percentages with ranges in parentheses.

† Significantly different from controls at $P < 0.05$ (Mann-Whitney non-parametric rank test).

Table 2. Lymphocyte subsets in bronchoalveolar lavage fluid (BALF) from control and maedi visna virus (MVV)-infected animals

	CD45	CD4	CD8	$\gamma\delta$ TCR	CD5	B cells	CD4/CD8 ratio
Control ($n=13$)	86.9* (68.4-96.7)	54.6 (42.3-67.1)	18.8 (8.5-30.4)	8.3 (3.8-19.6)	53.9 (40.6-66.9)	21.1 (13.1-51.1)	3.0 (1.8-5.3)
Maedi ($n=12$)	89.6 (73.3-98.9)	33.4† (10.6-67.1)	29.0† (2.9-68.3)	5.4 (1.8-25.8)	32.6† (10.7-57.6)	33.1 (11.9-92.6)	0.9‡ (0.3-4.0)

* Results are expressed as median of percentages of positively labelled cells in lymphocyte gate with ranges in parentheses.

† Significantly different from controls at $P < 0.05$ (Mann-Whitney non-parametric rank test).

‡ Significantly different from controls at $P < 0.001$ (Mann-Whitney non-parametric rank test).

Table 3. MHC class II expression on bronchoalveolar lavage fluid (BALF) macrophages from control and maedi visna virus (MVV)-infected animals

	Pan class II	VPM 37 (DR)	VPM 41 (DQ)
Control (n=13)	57.1* (10.5-233.7)	37.1 (5.87-51.26)	43.6 (9.4-195.1)
Maedi (n=12)	251.2† (41.3-827.3)	98.2‡ (33.2-242.3)	175.0† (39.8-270.0)

* Results are expressed as median of the value of mode channel number of fluorescence intensity with ranges in parentheses.
† Significantly different from controls at $P<0.05$ (Mann-Whitney non-parametric rank test).
‡ Significantly different from controls at $P<0.001$ (Mann-Whitney non-parametric rank test).

PB was similar for both groups and without significant statistical difference (control=0.88 (range 0.2-3.7); MVV=0.86 (range 0.3-2.2) (results expressed as median of percentages of positively labelled cells in lymphocyte gate)).

Macrophage phenotyping in BALF

Most of the macrophages expressed LCA (CD45 analogue), but neither the control nor the MVV-infected animals showed expression of CD4, CD8, $\gamma\delta$ TCR, CD5 or IgG light chain molecules on the alveolar macrophage population.

MHC class II expression on macrophages and lymphocytes

The results of the expression of the MHC class II on alveolar macrophages are shown in Table 3 and Fig. 1. As these molecules are constitutively expressed on most of the ovine alveolar macrophages [22] no significant differences in the percentages of cells expressing MHC class II between the two groups of animals were revealed. However, MVV-infected animals showed a higher fluorescence intensity when compared

with controls. By using the mode channel number, a significant increase in MHC class II molecule expression was observed on the alveolar macrophages of MVV-infected animals with the three reagents used. Similar studies on the lymphocyte populations of MVV-infected and control animals revealed no significant differences in the expression of these molecules (data not shown).

LFA-1 and LFA-3 expression on BALF lymphocytes and macrophages

The mode channel number was also used for the study of LFA-1 and LFA-3 as they were constitutively expressed on both lymphocytes and macrophages recovered from BALF. BALF lymphocytes from MVV-infected animals had significantly increased ($P<0.05$) LFA-1 (control=188.1 (range 47.7-311.9); MVV=255.8 (range 76.3-360.4)) and LFA-3 (control=42.8 (range 12.1-88.1); MVV=70.9 (range 51.3-126.5) (values expressed as median of the value of mode channel number)) (Fig. 2). Although alveolar macrophages from MVV-infected animals showed a higher expression of both molecules, no significant differences were found.

DISCUSSION

The characterization of the cellular phenotypes occurring in BALF during the course of MVV infection has not previously been described. The results shown in this study demonstrate alterations in the normal composition of CD4, CD8 and CD5 lymphocyte subsets and in the level of the expression of molecules that could play a key role in the pathogenesis of MVV in the lung and could provide a better understanding of the pathogenic mechanisms of lentiviruses.

The total BALF cell count in MVV-infected animals did not differ significantly from the controls. Considering that MVV lesions in lungs are characterized by an increased interstitial cellularity and a thickening of the alveolar septa [8] and also increased cellularity in BALF from MVV pathologically affected animals in slaughterhouses have been described [23],

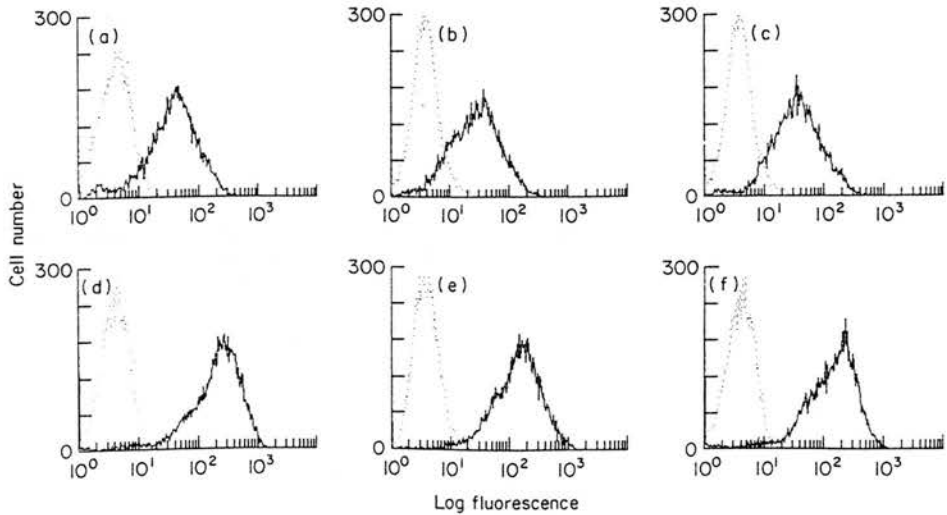


Fig. 1. Fluorescence histograms of MHC class II expression on bronchoalveolar lavage fluid (BALF) macrophages from control and maedi visna virus (MVV)-infected sheep. (a), (b), (c) Control animals. (d), (e), (f) MVV-infected sheep. (a), (d) Pan class II (VPM 36 and VPM 38). (b), (e) VPM 37 (DR). (c), (f) VPM 41 (DQ). (.....), Negative control. Fluorescence profiles are shifted to the right in MVV-infected animals for the three reagents used.

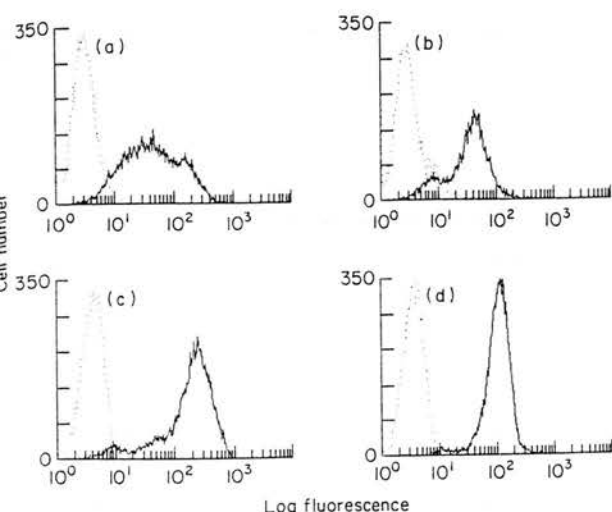


Fig. 2. Fluorescence histograms of LFA-1 and LFA-3 expression on bronchoalveolar lavage fluid (BALF) lymphocytes from control and maedi visna virus (MVV)-infected sheep. (a), (b) Control animals. (c), (d) MVV-infected sheep. (a), (c) LFA-1. (b), (d) LFA-3. (.....), Negative control. Fluorescence profiles are shifted to the right in MVV-infected animals in both cases.

These data could appear paradoxical. However, as we previously mentioned, our study is a cross section of an infected flock with animals in different stages of the disease (Collie *et al.*, manuscript accepted). This increase in the total cell count, even though not significant, seems to indicate a progressive tendency to an accumulation of cells in the lung interstitium.

A marked increase of BALF lymphocytes and neutrophils and a slight increase of macrophages in MVV-infected animals were the most noticeable results. Lymphocytes are the main mononuclear cell type found in MVV lung lesions [8] and an increased recovery of these cells can be expected. On the other hand, an increased secretion of Neutrophil Chemotactic Activity (NCA) by alveolar cells from MVV pathologically affected animals from slaughterhouses has been reported [23] and this could account for the increased recruitment of neutrophils into the affected lungs. However, the pathogenic role of these neutrophils, if any, remains to be determined. The increased percentage of neutrophils and lymphocytes seen in BALF of MVV-infected animals appeared to be a local phenomenon within the lung as none of these animals had elevated numbers of circulating neutrophils or lymphocytes. These findings are concordant with many other interstitial lung diseases in humans and animals [24].

The significant decrease ($P < 0.001$) in the $CD4^+/CD8^+$ ratio of lymphocytes from BALF of MVV-infected animals is due to both a significant increase of $CD8^+$ T lymphocytes and a significant decrease of $CD4^+$ T lymphocytes. Similar findings have been found in synovial fluid of sheep with MVV-induced arthritis [25] and in humans with AIDS-related lymphoid interstitial pneumonia [26]. However, the total depletion of $CD4^+$ T lymphocytes that characterizes AIDS and predisposes to opportunistic infections [27] is not found during the course of MVV infection, the $CD4^+$ T cell population being significantly decreased, but never absent. This finding would suggest that although lentiviruses both in humans and sheep induce inflam-

matory changes by similar mechanisms, they differ in some aspects of their pathogenesis. Further investigations are required to determine if the decrease in $CD4^+$ T lymphocytes reflects an infection in some cells of this lymphocytic subset. However, recent work demonstrated that sheep lentivirus is not lymphocytotropic and does not replicate productively in $CD4^+$ T lymphocytes obtained from blood and lymph [28]. Therefore, other explanations are needed for the $CD4^+$ T cell reduction. The decrease of $CD5^+$ cells seems to indicate lymphocyte activation as previous workers have reported an almost total loss of cell membrane $CD5^+$ expression during lymphocyte activation, as previous workers have reported an almost total infected animals. The biological role of these cells in the ruminant immune system remains unclear, although protection of epithelial surfaces has been proposed [29]. The fact that MVV does not involve epithelial damage in the lungs during its pathogenesis, could account for the lack of changes seen in $\gamma\delta$ T cells in BALF from MVV-infected animals.

Alterations in the distribution of lymphocyte phenotypes in BALF from MVV-infected animals were distinct from and not correlated with lymphocyte phenotype changes in PB. However, correlated changes between PB lymphocytes and synovial fluid lymphocytes in MVV-infected-cachectic animals have been observed [30]. It is known that blood lymphocytes do not necessarily reflect pathological changes in other organs/tissues [31] and previous studies have shown lack of correlation between alterations in lymphocyte subsets in blood and those in BALF [32].

Cell surface phenotypes of ovine bronchoalveolar macrophages from control and MVV-infected animals were similar, with expression of LCA and lack of $CD4$, $CD8$, $CD5$ and $\gamma\delta$ TCR. Similar results have been described in healthy sheep [22]. However, expression of $CD4$ and $CD8$ molecules on cultured ovine macrophages derived from blood monocytes from both MVV-infected and control animals has been observed (Wei-Cheng Lee, personal communication).

The two- to four-fold greater expression of MHC class II on alveolar macrophages of MVV-infected animals and the increased expression of LFA-1 and LFA-3 molecules on lymphocytes can account for the pathogenesis of the lymphoproliferative response and viral persistence. It has been demonstrated that class II antigens act as a component of the cellular receptor for MVV [33], and hence an increase of these molecules could promote the spread of the infection to uninfected macrophages, allowing persistence within the host. MVV-infected macrophages also induce the release of a specific interferon by T lymphocytes that is essential for the persistent expression of MHC class II antigens on the alveolar macrophages [34,35]. The continuous presence of these antigens could promote constant lymphocyte recruitment into the lungs, mediating local lesions and tissue damage. Furthermore, the increased expression of LFA-1 and LFA-3 in BALF lymphocytes of MVV-infected sheep would also contribute to the accelerated entry and accumulation of lymphocytes into the lung interstitium during the disease pathogenesis. LFA-1 and LFA-3 are cell adhesion molecules with a key role in leucocyte trafficking, activation and proliferation [36]. The increased expression of these molecules could have an important role as factors favouring the entrance and retention of new lymphocytes, their activation *in situ*, and their proliferation within the lungs, ending with the interstitial lymphoid proliferation seen in

the pulmonary parenchyma of MVV-infected animals. More studies are needed to understand the exact role of these and other adhesion molecules in the pathogenesis of the ovine lymphoid interstitial pneumonia.

ACKNOWLEDGMENTS

The authors acknowledge Mrs A. Baker and Mr A. Ross for their helpful collaboration and Dr J. Hopkins for critical reading of the manuscript. L.L. and I.B. are research fellows from the Spanish Ministerio de Educación y Ciencia (MEC) and the Spanish Instituto Nacional de Investigaciones Agrarias (INIA) respectively. This work was funded by AFRC Grant Number AG15/576 and by MEC.

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Effects on lung compliance, lung volume, and single-breath transfer factor for carbon monoxide in sheep with lentivirus-induced lymphoid interstitial pneumonia

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Summary

Static lung compliance, static lung volumes, and transfer factor for carbon monoxide were measured in 12 anesthetized adult Texel ewes seropositive for maedi-visna virus (MVV) and in 11 breed-, sex-, and age-matched seronegative controls. Median static lung compliance in MVV-infected sheep ($1.24 \text{ L} \cdot \text{kPa}^{-1}$; range, 0.27 to $2.20 \text{ L} \cdot \text{kPa}^{-1}$) was not significantly different from that in controls ($1.58 \text{ L} \cdot \text{kPa}^{-1}$; range, 0.82 to $2.08 \text{ L} \cdot \text{kPa}^{-1}$). Median body weight of MVV-infected sheep (56 kg ; range, 40 to 75 kg) was significantly ($P < 0.05$) less than that of controls (65 kg ; range, 53 to 87 kg). Median effective alveolar lung volume in MVV-infected sheep (3.36 L ; range, 1.44 to 4.52 L) was significantly ($P < 0.01$) less than that in controls (4.12 L ; range, 3.75 to 4.90 L). Median effective end expiratory lung volume in MVV-infected sheep (1.20 L ; range, 0.56 to 1.99 L) was significantly ($P < 0.001$) less than that of controls (1.98 L ; range, 1.76 to 2.78 L). Median lung volumes expressed per unit of body weight did not differ significantly between the groups. Median single-breath transfer factor for carbon monoxide in MVV-infected sheep ($7.89 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1}$; range, 3.45 to $12.74 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1}$) was significantly ($P < 0.001$) less than that in controls ($14.10 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1}$; range, 10.02 to $18.30 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1}$). Median transfer factor expressed per liter of alveolar volume in MVV-infected sheep ($2.44 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1} \cdot \text{L}^{-1}$; range, 1.28 to $3.72 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1} \cdot \text{L}^{-1}$) was significantly ($P < 0.05$) less than that in controls ($3.22 \text{ mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1} \cdot \text{L}^{-1}$; range, 2.47 to 3.74

$\text{mmol} \cdot \text{min}^{-1} \cdot \text{kPa}^{-1} \cdot \text{L}^{-1}$). These findings indicate that static lung volumes and transfer factor for carbon monoxide are significantly decreased in adult sheep naturally infected with MVV.

The principal pathologic feature of maedi-visna virus (MVV) infection in sheep is a slowly progressive lymphoid interstitial pneumonia¹ that causes dyspnea, reduced exercise tolerance, and loss of body condition.² The MVV shares considerable sequence homology and morphologic similarities with human immunodeficiency virus, also a lentivirus, the etiologic agent of the acquired immune deficiency syndrome (AIDS).³ Lymphoid interstitial pneumonia (LIP) is a recognized pulmonary complication of AIDS in adults⁴ and children⁵; however, the pathogenesis of LIP in people has yet to be elucidated, as have appropriate diagnostic and therapeutic measures.⁵ It is likely that the primary lymphoproliferative response seen in association with MVV-infection and with AIDS is a result of similar immunologic dysregulation.⁶ As a consequence, further investigation into the pathogenesis of MVV-infection is warranted.

Functional changes in human interstitial lung disorders are characterized by reduction in compliance, lung volumes, and transfer factor for carbon monoxide.^{7,8} Physiologic assessment of these functional changes, in combination with other investigative techniques, such as radiography, have proved useful in quantifying and monitoring the progression of LIP associated with AIDS in human beings.^{9,10}

Clinical signs of MVV infection are apparent only in the later stages of the natural disease, and little is known about disease progression in the preclinical phase. Meaningful investigation of the pathogenesis of naturally acquired maedi-visna requires the use of minimally invasive techniques to stage and monitor the course of disease. The use of physiologic techniques to measure lung function of infected sheep is a logical progression from clinical examination, which

Received for publication Apr 10, 1992.

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Supported by Agriculture and Food Research Council grant no. AG15/576.

The authors thank Mrs. A. Baker for technical assistance.

to identify sheep only in the latter stages of the disease.

To the authors' knowledge, lung functional changes associated with MVV infection have not been described; however, if present, these changes may be useful for staging the disease in the preclinical phase. Therefore, considered appropriate to examine sheep with natural MVV infection to characterize the changes of this disease on static and gas exchange properties of the lungs.

Materials and Methods

Sheep—Twelve adult Texel ewes (median body weight, 55 kg; range, 40 to 75 kg), seropositive for MVV on the basis of results of the agar gel immunodiffusion test,¹¹ and 11 breed-, age-, and sex-matched control ewes (median, 65 kg; range, 53 to 87 kg), seronegative for MVV, were studied. Body weight median values reflect the poorer body condition of seropositive sheep; however, both groups had similar skeletal conformation and were managed under similar conditions. The sheep had no evidence of respiratory tract disease unrelated to MVV infection as indicated by results of clinical examination, complete hematologic analysis, thoracic radiography, and necropsy examination for lungworm larvae by use of the modified Baermann technique.¹²

Animal preparation and anesthesia—General anesthesia was induced by IV administration of thiopentone sodium (20 mg/kg of body weight). Sheep were intubated with cuffed endotracheal tubes (9.5 mm ID, 15 mm OD) and positioned in sternal recumbency with the head supported on a cushioned rest. Esophageal pressure was measured by use of a latex balloon catheter (length, 15 cm; diameter, 1.5 cm; thickness, 0.05 mm), which was sealed over the end of a polyethylene catheter (3 mm ID, 4.5 mm OD), which in turn had a number of holes cut in spiral manner in the end sealed by the balloon. Prior to each series of measurements, the pressure-volume characteristics of the balloon-catheter assembly were validated according to the method of Senterre and Geubelle.¹³ The balloon was then evacuated, and 2 ml of air was added. The volume was shown to be within the range of compliance of this pressure recording system was close to the minimal relaxed volume of the balloon in air. The free end of the esophageal catheter was connected to a differential pressure transducer,^b and pressures were recorded on a strip chart recorder.^c Prior to passing the balloon catheter assembly, the distance from the nares to the middle of the thoracic portion of the esophagus was visually estimated, and this length was marked on the catheter to serve as initial validation of balloon position after insertion. The balloon was passed via the catheter and advanced until tracheal artifacts were absent from the tracings,¹⁴ negative deflections were observed in inspiration, and clearly defined cardiac

oscillations were discernible in the waveform. These precautions ensured that the balloon was placed in the midthoracic portion of the esophagus where the influences of periesophageal structures are minimal in cattle and goats.^{15,16} The other side of the differential pressure transducer was connected to a side port perpendicular to the airway opening, and the pressure thus recorded was defined as transpulmonary pressure (P_L). The pressure recording system was calibrated against a water manometer prior to each series of measurements.

If required, anesthesia was maintained, using an IV administered saline solution drip containing 0.20 to 0.25 mg of thiopentone sodium/kg infused at a rate of 2.0 to 2.5 ml/min.¹⁷ Sheep were ventilated, using a mechanical ventilator^d adjusted to maintain a tidal volume of 10 ml/kg and respiratory rate of 10 breaths/min.

Respiratory flow was measured, using a heated pneumotachograph^e connected to the end of the endotracheal tube. The pressure decrease across the pneumotachograph was measured, using a sensitive differential pressure transducer, and the signal was integrated to yield respiratory volumes,^b which were plotted on the chart recorder. The flow recording system was calibrated, using a 3-L syringe.

Static lung compliance (C_L)—After disconnecting the ventilator and allowing the sheep to passively expire to its relaxed functional residual capacity (FRC), the 3-L calibrated syringe was attached to the endotracheal tube and the lungs were inflated to P_L of 3 kPa. The volume of the lungs at P_L of 3 kPa was defined as total lung capacity (TLC). The sheep was then allowed to passively exhale to FRC; and the procedure was repeated twice to standardize the volume history of the lungs prior to measurements being made.¹⁸ To measure C_L , the lungs were again inflated to P_L of 3 kPa; however, the passive expiration was interrupted in stepwise manner by occluding the airway opening 8 to 15 times for 2- to 3-second intervals. By plotting P_L against expired volume, a static deflation curve was obtained. The slope of the linear portion of the curve from FRC to 40% of the way from FRC to TLC was determined by use of least-squares linear regression, and this value was taken as C_L , expressed in units of L·kPa⁻¹.

Lung volumes and transfer factor for carbon monoxide ($T_L CO$)—Lung volumes were measured, using a single-breath helium-dilution method performed in conjunction with the measurement of the transfer factor. A carbon monoxide and helium mixture (14% helium, 0.3% carbon monoxide, 85.7% air) was used to inflate the lungs from FRC to P_L of 3 kPa, at which point the airway opening was closed for 10 to 12 seconds. The valve was opened momentarily to allow washout of dead space, and thereafter, the expired alveolar gas was collected in an evacuated rebreathing bag. The concentration of helium in

^a Blue Line, Portex, Kent, United Kingdom.

^b CS9, Mercury Electronics, Glasgow, United Kingdom.

^c Inceis L6514, Belmont Instruments, Glasgow, United Kingdom.

^d Manley MN2, Hutchinson Bleas, Chesham, Bucks, United Kingdom.

^e Fleisch Ho. 2, Linton Instrumentation, Palgrave, Norfolk, United Kingdom.

Table 1—Means, medians, ranges, and coefficients of variation (CV) for pulmonary variables in control sheep (n = 11) and sheep with maedi-visna virus (MVV) infection (n = 12)

Measurement	Control				MVV-infected			
	Mean	Median	Range	CV (%)	Mean	Median	Range	CV (%)
Body weight (kg)	69.1	65.0	53 to 87	18	55.1	55.5*	40 to 75	15
C _L (L·kPa ⁻¹)	1.56	1.58	0.82 to 2.08	25	1.26	1.24	0.27 to 2.20	48
V _{A eff} (L)	4.31	4.12	3.75 to 4.90	9	3.30	3.37*	1.44 to 4.52	26
sV _{A eff} (ml·kg ⁻¹)	64	64	51 to 82	15	60	61	32 to 90	27
V _{EEL eff} (L)	2.09	1.98	1.76 to 2.78	14	1.31	1.20†	0.56 to 1.99	34
sV _{EEL eff} (ml·kg ⁻¹)	31	28	23 to 49	27	24	24	12 to 38	37
T _{L CO sb} (mmol·min ⁻¹ ·kPa ⁻¹)	14.02	14.10	10.02 to 18.30	16	8.15	7.89†	3.45 to 12.74	38
T _L /V _A (mmol·min ⁻¹ ·kPa ⁻¹ ·L ⁻¹)	3.24	3.22	2.47 to 3.74	11	2.50	2.44‡	1.28 to 3.72	30

* Median value significantly ($P < 0.01$) different from value for control sheep. † Median value significantly ($P < 0.001$) different from value for control sheep. ‡ Median value significantly ($P < 0.05$) different from value for control sheep.
C_L = static lung compliance; V_{A eff} = effective alveolar lung volume; sV_{A eff} = specific effective alveolar lung volume (ie, V_{A eff} expressed per unit of body weight); V_{EEL eff} = end-expiratory lung volume; sV_{EEL eff} = specific end-expiratory lung volume; T_{L CO sb} = single-breath transfer factor for carbon monoxide; and T_L/V_A = transfer factor per liter of alveolar volume.

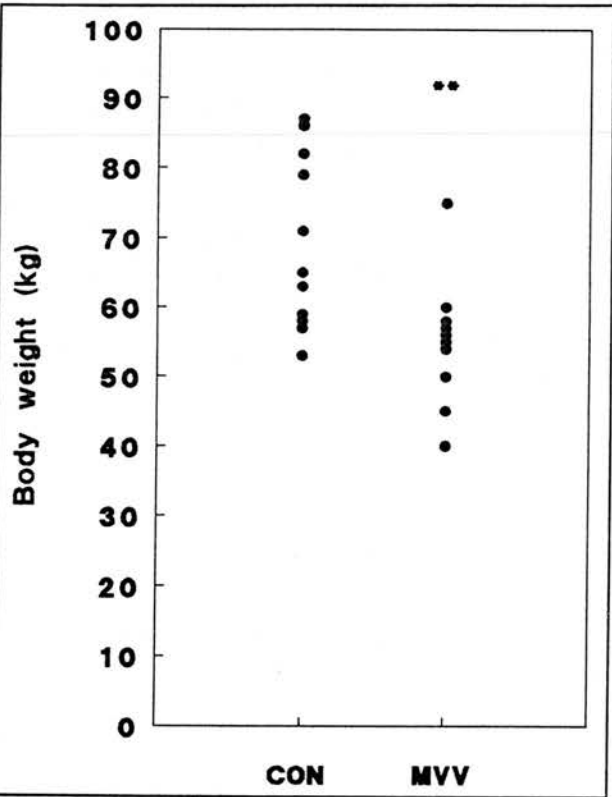


Figure 1—Body weight for 11 control (CON) and 12 maedi-visna virus (MVV)-infected sheep. ** Significantly ($P < 0.01$) lower than values for controls.

the bag was determined, using a katharometer.^f Dead space of equipment was estimated from internal dimensions, and anatomic dead space was estimated, using the appropriate allometric equation of Stahl,¹⁹ which relates anatomic dead space to body weight.

The effective alveolar volume (V_{A eff}) in liters (ie, the alveolar volume at which the breath was held

^f Resparameter Mk 4, PK Morgan, Chatham, Kent, United Kingdom.

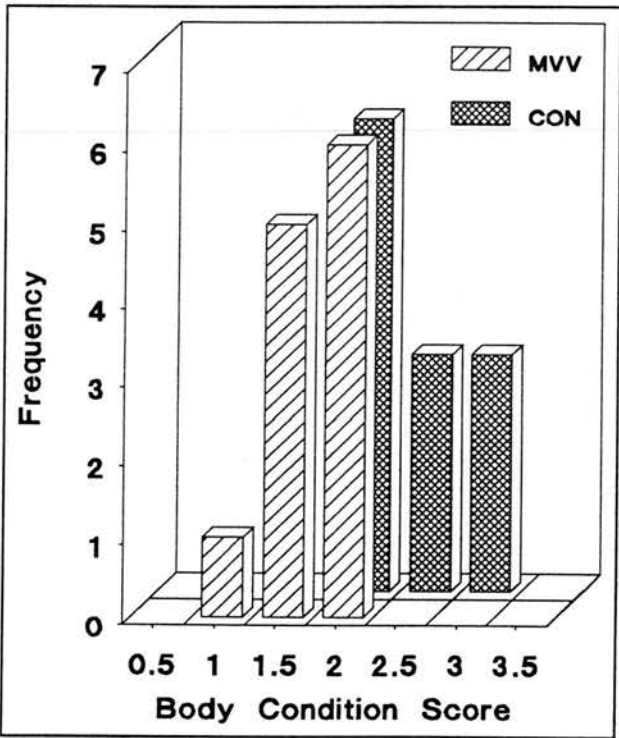


Figure 2—Histogram illustrating body condition scores for 11 CON and 12 MVV-infected sheep. Condition scores for the MVV-infected sheep were significantly ($P < 0.01$) less than the values for CON sheep.

minus the dead space of the airways) was calculated as follows:

$$V_{A \text{ eff}} = 1.05 \times (V_{\text{syrr}} - V_{D \text{ an}} - V_{D \text{ eq}}) \times (He_i/He_E)$$

where V_{syrr} = volume used to inflate lungs to P_L of 3 kPa, V_{D an} = anatomic dead space, V_{D eq} = instrumental dead space, He_i = helium concentration in the syringe, and He_E = helium concentration in the expired sample. The coefficient 1.05 is a factor used to account for a change in He_E attributable to carbon

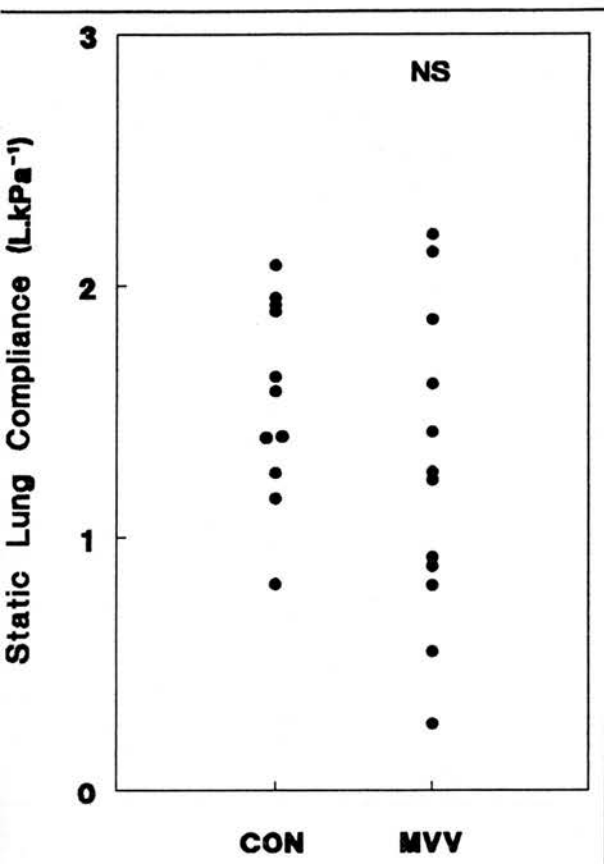


Figure 3—Static lung compliance values ($\text{L}\cdot\text{kPa}^{-1}$) for 11 CON and 12 MVV-infected sheep. NS = no significant difference between the groups.

dioxide absorption during breath-holding.²⁰ By subtracting V_{sy} from $V_{\text{A eff}}$, an estimate of the lung volume at the end-expiratory level ($V_{\text{EEL eff}}$) was obtained.

The single-breath transfer factor for carbon monoxide ($T_{\text{L CO sb}}$) was determined, using a modification of the technique described by Karp et al.²¹ The technique involves 10-seconds of breath-holding of a carbon monoxide and helium in air gas mixture, with subsequent collection of an alveolar gas sample. The concentration of carbon monoxide in the syringe initially and in the alveolar gas sample, together with the time of breath-holding and the alveolar volume during breath-holding, are used in the calculation of $T_{\text{L CO sb}}$ as follows:

$$T_{\text{L CO sb}} = 53.6 \times V_{\text{A eff}} \times t^{-1} \times \log_{10} \left(\frac{\text{CO}_1 \cdot \text{He}_E \cdot \text{CO}_E^{-1} \cdot \text{He}_1^{-1}}{\text{CO}_1 \cdot \text{He}_E \cdot \text{CO}_E^{-1} \cdot \text{He}_1^{-1}} \right)$$

where t is the time of breath-holding in seconds, $V_{\text{A eff}}$ is the effective alveolar volume, and He_E and CO_E are end-tidal alveolar concentrations and He_1 and CO_1 are syringe concentrations of helium and carbon monoxide respectively.²² Units of $T_{\text{L CO sb}}$ are expressed as $\text{mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$. Because the transfer factor is positively correlated with the lung volume at which the measurement is made, it is also expressed per liter of alveolar volume (ie, $T_{\text{L}}/V_{\text{A}}$).

During the period of anesthesia, a blood sample was collected and hemoglobin concentration was

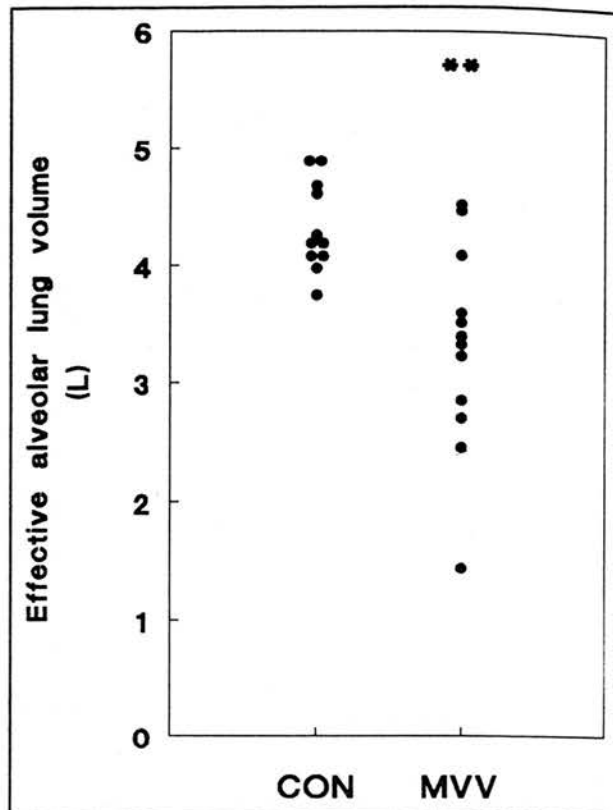


Figure 4—Effective alveolar lung volume values for 11 CON and 12 MVV-infected sheep. See Figure 1 for key.

measured by use of an automated analyzer.⁸ This concentration of hemoglobin was used in the equation of Cotes,²³ to adjust observed $T_{\text{L CO sb}}$ to a standard hemoglobin concentration of 14.6 g/dl.

Analysis of data—Three determinations of C_{L} lung volumes, and transfer factor were made on each sheep, and the mean value was calculated. Absolute lung volumes were also expressed per unit of body weight (ie, specific end expiratory lung volume [$sV_{\text{EEL eff}}$] and specific effective alveolar volume [$sV_{\text{A eff}}$]). The values recorded for the MVV-seropositive and -seronegative groups of sheep were statistically compared, using the Mann-Whitney test. Linear regression analysis with ANOVA was used to examine the relation between C_{L} and body weight and C_{L} and $V_{\text{A eff}}$ for each group.

Results

Mean, median, range, and coefficients of variation were determined for measurements of body weight, C_{L} , absolute and specific lung volumes, transfer factor, and transfer factor expressed per unit of alveolar volume for each group of sheep (Table 1).

The 95.5% confidence interval for the difference in population medians for body weight ranged from -26 to -3 kg. This difference was statistically significant ($P < 0.01$; Fig 1). Body condition scores for

⁸ Baker System 9000, Serono Baker Diagnostics, Allentown, Pa.

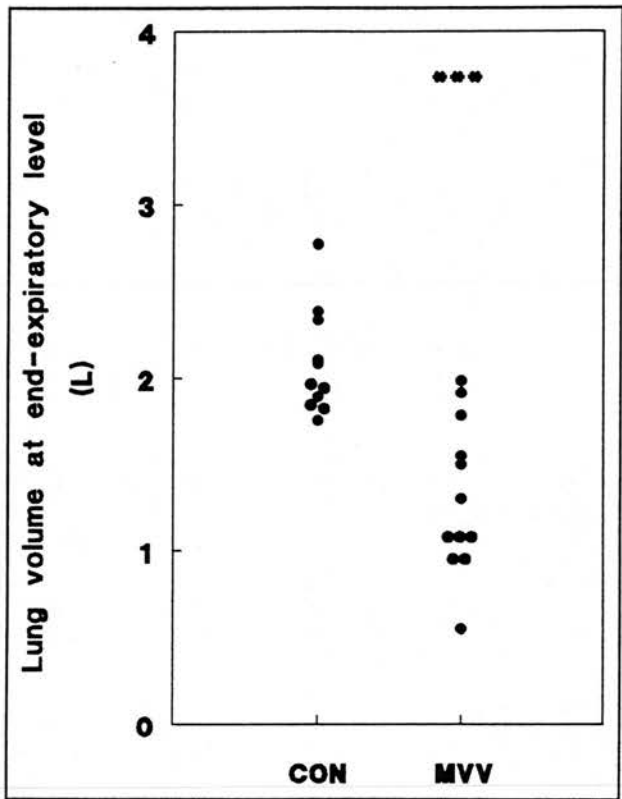


Figure 5—Lung volume values at end-expiratory level for 11 CON and 12 MVV-infected sheep. *** Significantly ($P < 0.001$) lower than values for controls.

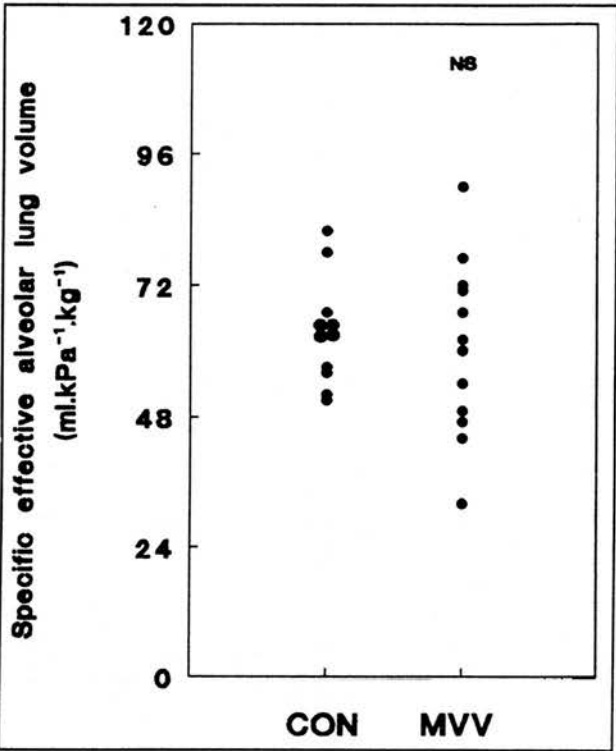


Figure 6—Specific effective alveolar lung volume values for 11 CON and 12 MVV-infected sheep. See Figure 3 for key.

MVV-infected sheep were significantly ($P < 0.01$) less than scores for controls (Fig 2).

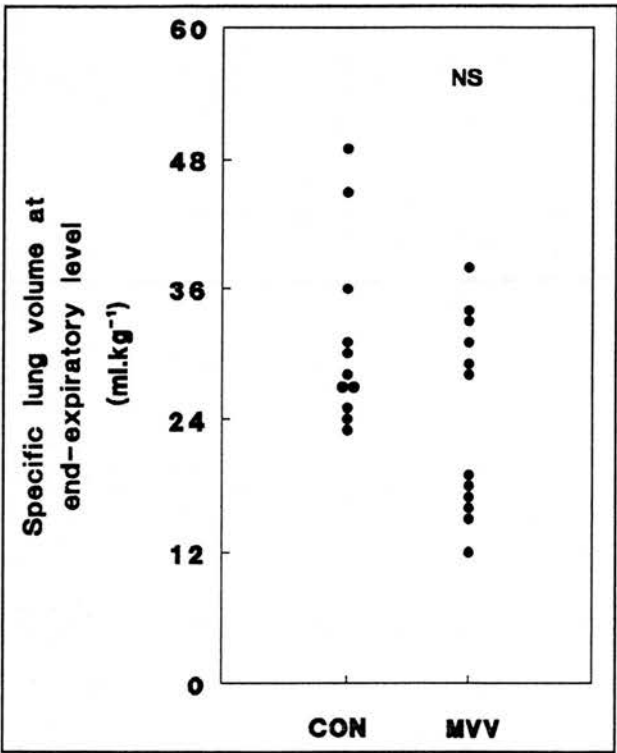


Figure 7—Specific end-expiratory level lung volume values for 11 CON and 12 MVV-infected sheep. See Figure 3 for key.

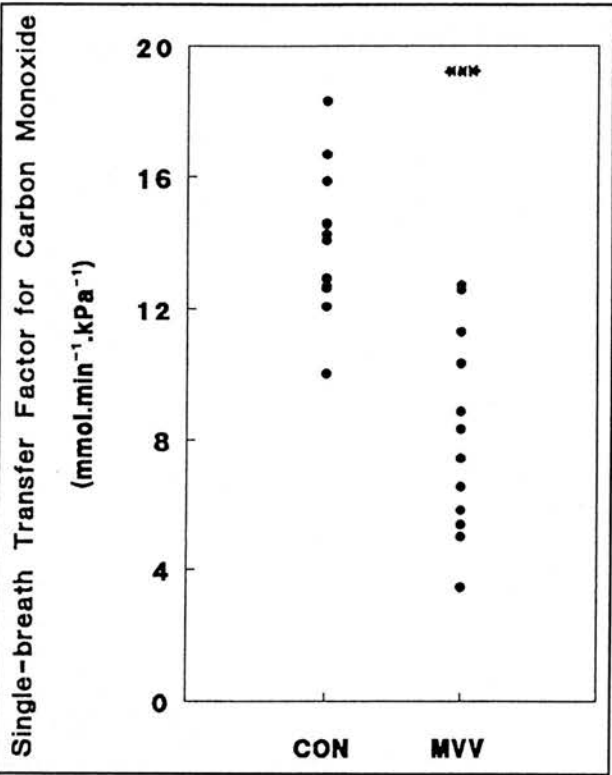
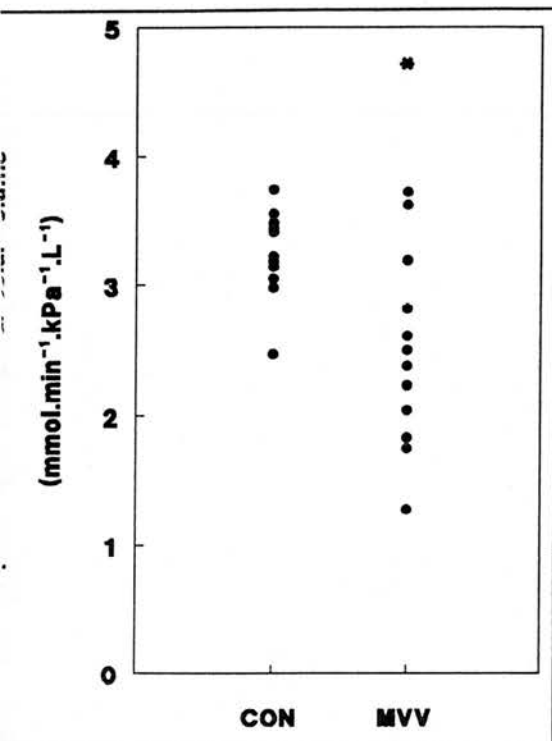
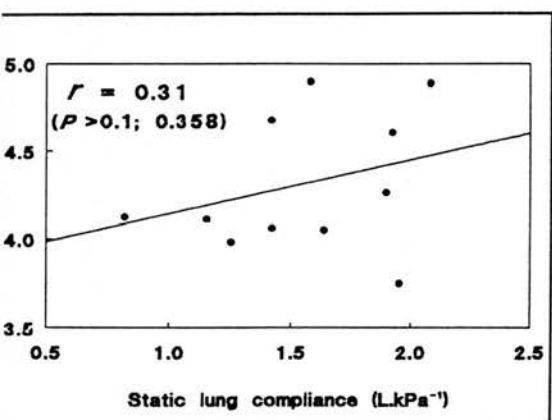


Figure 8—Single-breath transfer factor for carbon monoxide values for 11 CON and 12 MVV-infected sheep. See Figure 5 for key.

The 95.5% confidence interval for the difference in population medians for C_L ranged from -0.76 to 0.19 L.kPa^{-1} (Fig 3). This difference was not statistically significant.



9—Transfer factor values expressed per liter of effective alveolar volume for 11 CON and 12 MVV-infected sheep. Significantly ($P < 0.05$) lower than values for controls.



10—Relation between effective alveolar volume and static lung compliance for control sheep. The solid line represents the line of best fit by least-squares linear regression.

The 95.5% confidence interval for the difference in population medians for $V_{A\text{ eff}}$ ranged from -1.53 to 0.43 L (Fig 4). This difference was statistically significant ($P < 0.01$).

The 95.5% confidence interval for the difference in population medians for $V_{EEL\text{ eff}}$ ranged from -1.09 to 0.40 L (Fig 5). This difference was statistically significant ($P < 0.001$). Specific lung volume values were determined for individual sheep (Fig 6 and 7). Differences between groups were not statistically significant for $sV_{A\text{ eff}}$ and $sV_{EEL\text{ eff}}$.

The 95.5% confidence interval for the difference in population medians for transfer factor values ranged from -8.44 to -3.28 $\text{mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$ (Fig 8). This difference was statistically significant ($P < 0.001$).

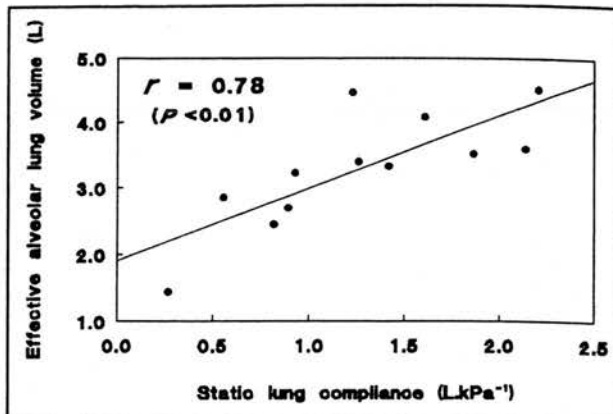


Figure 11—Relation between effective alveolar volume and static lung compliance for MVV-infected sheep. See Figure 10 for key.

Transfer factor values were also expressed per unit of alveolar volume (Fig 9). The 95.5% confidence interval for the difference in population medians for T_L/V_A ranged from -1.32 to -0.22 $\text{mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}\cdot\text{L}^{-1}$. This difference also was statistically significant ($P < 0.05$).

The relation between effective alveolar volume and C_L was determined for control (Fig 10) and MVV-infected (Fig 11) sheep. Results of least-square regression analysis relating C_L to body weight and $V_{A\text{ eff}}$ to C_L were tabulated for control and MVV-infected sheep (Table 2).

Discussion

The history of the flock from which these MVV-infected sheep were derived has been described.²⁴ Postmortem examination of sheep from the parent flock have indicated that lungs with diffuse and extensive MVV lesions have a characteristic gross appearance.²⁴ On opening the thoracic cavity, the lungs tended not to collapse, appearing larger than normal. Lung weight was increased (0.52 to 1.96 kg; normal 0.4 to 0.6 kg), and the lungs were firm when cut and were rubbery. Consistent histopathologic features were smooth muscle hyperplasia and interstitial infiltration with lymphocytes, monocytes, macrophages, and some plasma cells.

Although all sheep of this study were adult, it is acknowledged that factors including age and time and route of infection would differ between individual sheep and, as such, they would probably have a spectrum of pulmonary pathologic features. Attempt was not made to grade or classify these sheep on a clinical basis; they represented a cross-section of the adult sheep within the flock.

The body weight and body condition score of MVV-infected sheep were significantly lower than values for the control sheep. With loss of body condition being a frequent early sign of MVV infection,^{2,25} it is assumed that these observations are a feature of the disease process. Nutritional management of the 2 groups of sheep was identical and intercurrent disease was not apparent in either group on the basis of results of clinical or laboratory investigation. Weight

Table 2—Least-squares regression equations relating static lung compliance (C_L) to body weight (BW) and effective alveolar volume to C_L for control (CON) and MVV-infected sheep

Group	Dependent variable (unit)	Independent variable (unit)	Regression equation	F	r^2 (%)	P
CON	Static lung compliance (L·kPa ⁻¹)	Body weight (kg)	$0.414 + 0.0166 \times BW$	3.51	28.1	0.094
MVV	Static lung compliance (L·kPa ⁻¹)	Body weight (kg)	$-0.49 + 0.0318 \times BW$	2.44	19.6	0.149
CON	Effective alveolar volume (L)	Static lung compliance (L·kPa ⁻¹)	$3.83 + 0.310 \times C_L$	0.94	9.4	0.358
MVV	Effective alveolar volume (L)	Static lung compliance (L·kPa ⁻¹)	$1.89 + 1.11 \times C_L$	15.55	60.9	0.003

The coefficients of determination (r^2) in percentage units, F-ratios (F), and significance of the association between the variables (P) are shown.

loss and cachexia were commonly observed in the flock of origin.²⁴

The normal pulmonary interstitium is composed of connective tissue components (collagen, elastic fibers, proteoglycans, and fibronectin), mesenchymal cells, and inflammatory and immune effector cells.²⁶ The most obvious physical effect of LIP is a thickening of the interstitial space, with consequent alteration of the number, form, and location of its cellular and noncellular elements. The anticipated physiologic consequences of such structural alterations include changes in compliance, lung volumes, and transfer factor. Because the pressure-volume characteristics of the lung are principally determined by the tissue network of collagen and elastic fibers (ie, the noncellular elements in the interstitium),²⁷ any change to this network is liable to lead to changes in compliance. Static lung volumes are influenced by lung and thoracic wall recoil pressures²³ and the volume of tissue and fluid within the thoracic cavity.²⁸ Changes in both determinants would be anticipated in LIP.

Defined as the rate of transfer of a gas per unit driving pressure between the alveoli and the erythrocytes within the pulmonary capillaries, transfer factor is affected by a number of structural lung dimensions, including lung volume, the path length for diffusion in the gas phase, the surface area of the alveolar capillary membrane, and the volume of blood in the capillaries perfusing ventilated alveoli.²² Changes to several of these dimensions occur in LIP; thus, alterations in transfer factor would be anticipated.

The single-breath helium dilution method is commonly used to measure $V_{A\text{ eff}}$ in human beings, though it does tend to underestimate the true lung volume in subjects with airflow restriction.²⁰ However, for subjects with only slight airflow limitation or interstitial lung disease, use of this technique is accepted.²⁹ The use of the derived measurement, $V_{EEL\text{ eff}}$, is subject to the same limitations.

Use of the equation of Cotes²² to correct transfer factor values for differences in hemoglobin concentration between individual sheep assumes that the reaction rates of carbon monoxide with sheep and human oxyhemoglobin are similar.

Fisher and Hyde³⁰ documented that pulmonary

diffusing capacity and capillary blood flow were significantly reduced during passive lung inflation and breath-holding in anesthetized dogs. The cause of this reduction was attributed to decreased venous return to the right side of the heart and increased pulmonary vascular resistance. The magnitude of this reduction was significantly correlated with the level of transthoracic pressure used to induce lung inflation. In addition, a temporal change in diffusing capacity was detected, with a significant decrease in the first 7 seconds and no significant change between 7 and 11 seconds.³⁰ Therefore, it is necessary, when comparing single-breath transfer factor values between animals, to control lung inflation pressures and duration of breath-holding as was done in the sheep of this study.

The range of compliance values was wide for control and MVV-infected sheep. Halmagyi and Colebatch¹⁷ studied anesthetized sheep (n = 27) with mean \pm SD body weight of 42.6 ± 4.7 kg and reported dynamic lung compliance of 1.08 ± 0.316 L·kPa⁻¹. By expressing this value per unit of body weight, an approximate value of $25 \text{ ml} \cdot \text{kPa}^{-1} \cdot \text{kg}^{-1}$ is obtained. This is in reasonable agreement with the value of $22.7 \text{ ml} \cdot \text{kPa}^{-1} \cdot \text{kg}^{-1}$ for our control sheep. In their series, Halmagyi and Colebatch obtained an intersubject coefficient of variation of 29% for measurements of dynamic compliance and commented that this degree of scatter was not dissimilar to that obtained in human beings. The coefficient of variation between control sheep for compliance measurements in this study was 25%; thus, intersubject variability was comparable to previous observations in anesthetized sheep. One factor that might contribute to this variation is body weight. Although compliance was positively correlated with body weight in control and MVV-infected sheep, the relation was not statistically significant as assessed by linear regression. Analysis of variance indicated that body weight accounted for only 19.6% of the variation in compliance in MVV-infected sheep and for 28.1% in the control sheep.

Given the observation that MVV-affected lungs tend not to collapse when removed from the thorax,²⁴ suggesting low compliance, lack of any significant difference between the groups was unexpected. Al-

gh significant difference was not observed between the groups as a whole, the range of values was greater for the MVV-infected sheep with 5 individual sheep having $C_L < 1 \text{ L} \cdot \text{kPa}^{-1}$. Thus, changes in compliance may be a functional change only detectable at a particular stage of the disease, and perhaps if a more critical selection procedure had been used to select sheep (eg, on a clinical or age-related basis), significant differences would have been observed between MVV-infected and control sheep.

Changes in compliance in people with interstitial lung disease seem to reflect predominantly sarcoidosis³¹ and are not correlated with inflammatory lesions.³² In visna and maedi, the predominant lesions are cellular infiltration of the interstitium together with distinct smooth muscle hyperplasia. Although the exact pathophysiologic role of the smooth muscle hyperplasia is unknown, one theory is that it may be to compensate for the lack of elastic recoil resulting from the thickening of the interalveolar septa, fragmentation and destruction of elastic fibers in the disease.¹ Accordingly, measurements of compliance in MVV-infected animals will presumably be influenced by the quantity and functional tone of smooth muscle in the lungs.

Previously reported³³ static lung volume values (TLC of $76 \text{ ml} \cdot \text{kg}^{-1}$ and FRC of $25 \text{ ml} \cdot \text{kg}^{-1}$) in anesthetized sheep compare with values of $64 \text{ ml} \cdot \text{kg}^{-1}$ for $V_{A \text{ eff}}$ and $31 \text{ ml} \cdot \text{kg}^{-1}$ for $V_{EEL \text{ eff}}$ in our study. The differences in these measurements likely result from the effects of anesthesia and different methods used in these studies. Intersubject coefficients of variation of 10 to 15% were reported³³ for static lung volume measurements and compare favorably with intersubject coefficients of variation of 9% for $V_{A \text{ eff}}$ and 14% for $V_{EEL \text{ eff}}$.

Lung volumes were significantly lower in MVV-infected sheep than in controls; however, when corrected for body weight, significant difference between the groups could not be detected. It might be argued that the difference in lung volumes between the groups is simply a reflection of different body sizes, with a normal ratio of lung volume to body weight being maintained in the smaller MVV-infected sheep. However, our subjective opinion that sheep of both groups had similar skeletal dimensions coupled with the significantly poorer condition scores of MVV-infected sheep, counters this argument and suggests that there is a concomitant decrease in body weight and lung volume as a result of the disease process. To support this argument, that smaller lung volumes of the diseased sheep are a function of the disease process, is the observation that compliance shares a significant linear relation with lung volume in MVV-infected sheep ($r = 0.78$; $P < 0.01$), whereas no such relation can be documented for the control sheep ($r = 0.31$; $P > 0.1$). Indeed, compliance accounted for 60.9% of the variation in lung volume in MVV-infected sheep and only accounted for 9.4% in the control sheep. In contrast, body weight accounted for 33.3% of the variation in lung volume in the control sheep and for only 11.3% in MVV-infected sheep. Thus, a major influence on

lung volume in MVV-infected sheep would appear to be compliance.

Similar changes in lung volumes are recognized in human beings with idiopathic pulmonary fibrosis, in whom volume reduction may also be accompanied by a decrease in lung compliance.³⁴ A reduction in lung volume is also a common feature of sarcoidosis³⁵ and may be seen in people with LIP.^{10,36}

Transfer factor per se and transfer factor corrected for alveolar lung volume values were significantly reduced in MVV-infected sheep. This is a common finding in human beings with interstitial lung disease²⁶ in whom transfer factor measurements may be useful for the follow-up evaluation of individual cases of sarcoidosis and interstitial fibrosis.³⁷ The $V_{A \text{ eff}}$ value is the single greatest source of variability of $T_{L \text{ CO sb}}$ ²⁹ with a decrease in $V_{A \text{ eff}}$ exposing a smaller alveolar membrane surface area to participate in gas exchange. The significantly lower $V_{A \text{ eff}}$ in MVV-infected sheep may, therefore, contribute to the observed difference in $T_{L \text{ CO sb}}$. That other factors are involved in the reduction of the transfer factor in MVV-infected sheep is evidenced by the still significant reduction in transfer factor after correction for lung volume. Such factors are likely to include alterations in ventilation-to-perfusion ratio, pulmonary hypoperfusion, capillary transit time, and thickening and functional alterations of the interalveolar membrane brought about by the cellular infiltration.³⁸

In people with interstitial lung disease, ventilation-perfusion inequalities account for the major part of observed hypoxemia.³⁴ These inequalities, which are thought to arise as a result of nonuniform alterations to the mechanical properties of the lung parenchyma,³⁹ can reduce the effective interface for gas transfer and complicate the interpretation of transfer factor measurements made using steady-state methods.²³ However, for the single-breath technique, where a uniform distribution of the inspirate can be anticipated, maldistribution of ventilation to perfusion is unimportant unless it is extreme.⁴⁰ Other unequal distributions (eg, the distributions of diffusion properties relative to alveolar volumes and pulmonary capillary blood flow throughout the lung), are likely present in MVV-infected sheep, and may reduce apparent single-breath transfer factor values with increasing time of breath-holding.⁴⁰ Despite these latter limitations, single-breath transfer factor values should provide a useful numerical index of the overall transfer characteristics of the lungs in MVV-infected animals.

These physiologic changes, specifically reduction in lung volumes and transfer factor, are consistent with those found in people with interstitial lung disease.^{7,8} Knowledge that measurable changes in lung function do occur in natural MVV infection provides a basis for further investigation of the potential of lung function studies as a means of staging this disease.

Sheep are useful experimental animals in pulmonary research—their lungs being of comparable size to those of human beings.⁴¹ In addition, sequential analysis of the bronchoalveolar milieu of con-

scious sheep by physiologic, histologic, and bronchoalveolar measurements is well tolerated, with reproducibility and variations of measurements being similar to those found in human beings.³³ Accordingly, integration of these techniques with recently developed immunologic and molecular biological technologies will provide a powerful means for investigating the pathogenesis of LIP induced by ovine lentivirus infection.

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Lung compliance, lung volume and transfer factor for carbon monoxide in anaesthetised sheep: normal values and reproducibility of measurements

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Measurements of quasistatic compliance (C_{qst}), effective alveolar volume ($V_{A,eff}$) and single-breath transfer factor for carbon monoxide ($T_{L,CO}$, 'sb') were completed in 16 normal, anaesthetised, adult Texel ewes. Regression equations were computed for these variables as a function of bodyweight and the optimal equations selected. The 95 per cent prediction intervals for the equations were calculated such that normal lung function in similar sheep could be accurately predicted. The long term reproducibility of these measurements was assessed in nine sheep, measured at monthly intervals over a period of five months. Although measurements made in individual sheep were often highly variable, the variation between repeated measurements on the separate days for the group was insignificant.

In human respiratory research, lung function data are frequently assessed in relation to predicted values for the subject under study. These predicted values are generated using multiple regression equations previously calculated for a population similar to that from which the subject was obtained. The best reference (independent) variables used for humans are age, sex, stature and ethnic group, with these variables accounting for 50 per cent of the total variability about the regression lines (Cotes 1979). In comparative respiratory research, Stahl (1967) demonstrated, using data available from the literature, the relationship of several respiratory variables to bodyweight over a wide range of mammalian species. Such predictions, based on values over such a large weight

range, may not agree with studies on smaller groups of animals of a particular species (Stahl 1967) where social and environmental factors may lead to physiological adaptation. Indeed in humans, bodyweight is not the anthropometric variable of choice due to large differences within populations regarding the contribution of adipose tissue and muscle in individuals (Cotes 1979).

In sheep, where breed, age, sex and environmental and social factors may influence body condition and hence bodyweight, reference to such predictions of respiratory variables based solely on bodyweight should be cautious. For this reason, where the study of respiratory variables in a particular category of sheep is planned, predicted normal values should ideally be generated using normal sheep of similar description.

Certain routine pulmonary function tests applied to humans can show considerable intra-subject variability (Guyatt et al 1975, Hutchison et al 1981, Pennock et al 1981) and similar variability is seen when comparable lung function measurements are applied to trained conscious sheep (Begin et al 1981) and cattle (Gallivan and McDonnell 1988). Although this variability can preclude the use of these measurements to monitor individual animals over time, the use of groups of animals can overcome this limitation (Gallivan and McDonnell 1988).

Whether for ethical or procedural reasons, circumstances may dictate that lung function measurements cannot be undertaken in naive conscious animals and in these situations anaesthesia is a frequently considered alternative. However, Southorn et al (1980) demonstrated that anaes-

can contribute to intrasubject variability of pulmonary mechanics and static lung volume measurements in dogs. This variable effect of anaesthesia on pulmonary function measurements in individuals will presumably occur in other species and may limit their potential for monitoring changes in lung function in groups of animals over time.

The purposes of this study were, first, to generate regression equations relating pulmonary function variables to bodyweight for anaesthetised Texel sheep such that normal values could in future be generated for sheep of similar breed, age, sex, managed under similar conditions, and, secondly, to examine the long term reproducibility of lung function measurements obtained during anaesthesia.

Materials and methods

Animals

Seventeen adult Texel ewes (bodyweight 52 to 87 kg) were used. All the animals were free from clinically apparent cardiopulmonary dysfunction. Routine examinations including routine haematology, thoracic radiography, faecal examination for lungworm larvae and serological testing for absence of maedi-visna virus infection, confirmed absence of significant cardiopulmonary dysfunction. The sheep were housed during the period of measurements.

Anaesthesia

Food was withheld for 12 hours before anaesthesia which was achieved by intravenous administration of a single bolus of thiopentone sodium at a rate of 20 mg kg⁻¹ bodyweight. The sheep were weighed on the morning of the procedure. Following induction the sheep were intubated with cuffed endotracheal tubes (diameter 9.5 to 11 mm) and placed in sternal recumbency with head supported on a cushioned rest. They were ventilated with medical air (BOC) using a mechanical ventilator (Manley MN2, Hutchinson Blease) set to maintain a tidal volume of 10 ml kg⁻¹ bodyweight and respiratory rate of 10 breaths min⁻¹. To facilitate the measurement of respiratory flows a heated pneumotachograph (Fleisch number 2, Linton Instrumentation) was connected to the end of the endotracheal tube and the pressure drop across the pneumotachograph was mea-

sured using a sensitive differential pressure transducer (CS9, Mercury Electronics) with the signal subsequently integrated to yield respiratory volumes. Changes in transpulmonary pressure (ΔP_{tp}) were measured using a differential pressure transducer (CS9, Mercury Electronics). One side of this transducer was connected via a polyethylene catheter (3 mm inner diameter, 4.5 mm outer diameter) to a latex balloon (length 15 cm, diameter 1.5 cm) sealed over the distal end of the catheter and placed in the caudal third of the thoracic oesophagus. The balloon was then evacuated and 2 ml of air was added. This volume was shown to be within the range of high compliance of this pressure recording system and was close to the minimal relaxed volume of the balloon in air. The other side of this transducer was connected to a side port perpendicular to the airway opening.

Outputs from pressure and flow measuring devices were recorded on a strip chart recorder (Linseis L6514, Belmont Instruments). Before measurements were made, the pressure recording system was calibrated against a water manometer and the integrated volume recording was calibrated using a 3 litre syringe.

Lung function measurements

Quasistatic compliance (C_{qst}). Following cessation of spontaneous respiratory efforts (within two to three minutes of commencing mechanical ventilation in every case) the sheep were disconnected from the ventilator and allowed to exhale passively to functional residual capacity (FRC). The lungs were then actively inflated to $\Delta P_{tp} = 3$ kPa using the 3 litre calibrated syringe filled with room air and immediately thereafter allowed to deflate passively to FRC. This procedure was repeated twice to stabilise lung volume history before measurements of quasistatic compliance were made. The volume of the lungs at $\Delta P_{tp} = 3$ kPa was defined as total lung capacity (TLC). To measure C_{qst} , the lungs were again inflated to TLC, however the passive exhalation was interrupted in a stepwise fashion by occluding the airway opening eight to 15 times for two to three second intervals. By plotting ΔP_{tp} against expired volume a quasistatic deflation curve was obtained. The slope of the linear portion of the curve from FRC to 40 per cent of TLC was determined by least squares linear regression and this value taken as C_{qst} , expressed in units of litres kPa⁻¹. To achieve adequate data

for the least squares regression, the measurement procedure outlined above was repeated with pooling of data points such that a single 'procedure' value of C_{qst} for each sheep was obtained.

Alveolar volume ($V_{A,eff}$) and transfer factor for carbon monoxide ($T_{L,CO}$)

Using a single-breath helium dilution technique, and $T_{L,CO}$, using a forced single-breath technique, were measured concurrently as the same procedure. Following a period of mechanical ventilation the sheep were disconnected from the ventilator and allowed to exale passively to FRC. The 3 litre calibrated syringe was used to inflate the lungs to $\Delta P_{tp} = 3$ kPa with a monoxide and helium in air gas mixture (10 per cent helium, 0.3 per cent carbon monoxide, 99.7 per cent air; BOC). A valve at the airway was closed and this gas mixture was held in the lungs for a period of 10 to 12 seconds. The valve was then opened momentarily to allow washout of dead space before the remaining gas in the lungs was collected in an evacuated rebreathing bag. The concentration of helium in this expired gas sample was measured using a gas analyser (Resparameter Mk 4, P. K. Morgan). $V_{A,eff}$ in litres, that is, the alveolar volume at the end of the breath was held minus the dead space of the lungs and equipment was calculated as follows:

$$V_{A,eff} = 1.05 \cdot (V_{syr} - V_{D,an} - V_{D,eq}) \cdot (He_I/He_E)$$

V_{syr} = volume used to inflate lungs to ΔP_{tp}
 $V_{D,an}$ = anatomical dead space, $V_{D,eq}$ = equipment dead space, He_I = helium concentration in the syringe and He_E = helium concentration in the expired sample (Quanjer et al 1983). Equipment dead space was calculated from inter-breath measurements and the anatomical dead space was calculated using the equations of Stahl (1967) which relate respiratory variables to bodyweight in different species.

Single-breath transfer factor for carbon monoxide ($T_{L,CO, 'sb'}$) was calculated as follows:

$$T_{L,CO, 'sb'} = 53.6 \cdot V_{A,eff} \cdot t^{-1} \cdot \log_{10} (CO_I \cdot He_E \cdot CO_E^{-1} \cdot He_I^{-1})$$

t = time of breathholding in seconds and CO_E and CO_I were alveolar concentrations, and CO_I and CO_E were syringe concentrations of helium and carbon monoxide, respectively (Cotes 1983). Time

of breathholding was calculated according to the recommendations of the Epidemiology Standardisation Project (ESP) (Ferris 1978). A blood sample was taken during the period of anaesthesia and haemoglobin concentration measured using an automated analyser. $T_{L,CO, 'sb'}$ values were then adjusted to a standard haemoglobin concentration of 14.6 g dl⁻¹ according to the method of Cotes (1979). $T_{L,CO, 'sb'}$ is expressed in units of mmol min⁻¹ kPa⁻¹. Since the transfer factor is positively correlated with the lung volume at which the measurement is made, it was also expressed per litre of alveolar volume, that is, T_L/V_A . The mean value of three determinations of $V_{A,eff}$ and $T_{L,CO, 'sb'}$ was calculated and used in the statistical analyses. The average duration of the completed series of measurements was approximately 20 minutes.

Experimental design and statistical analysis

Long term reproducibility of the lung function data was examined by repeating measurements in the same group of nine sheep at monthly intervals over a period of five months. A two-way mixed model analysis of variance (ANOVA) without replication was used to estimate the between day variability of lung function variables for the group. In this analysis, the individual sheep are considered the random factor and the time dimension a fixed treatment effect (Sokal and Rohlf 1981). A Kolmogorov-Smirnov test was used to test for normal distribution of the pooled error terms in each ANOVA and a Bartlett's test was used to check for homogeneity of variances between samples in each ANOVA (Sokal and Rohlf 1981). Data from each ANOVA satisfied both of the above preconditions. The coefficient of variation (CV) for the repeated measurements of each variable in individual sheep was also calculated.

The relationship of individual lung function variables to bodyweight was examined for all 16 sheep. Lung function and bodyweight measurements were made at least three times on each sheep with the interval between measurements being between 30 and 77 days. The data from a total of 70 procedures were used in the analysis. Regression analyses based on linear, polynomial and allometric regression equations were executed to determine the relationship between bodyweight (the independent variable) and the lung function data (the dependent variable). Using the most statistically significant regression equations, 95 per

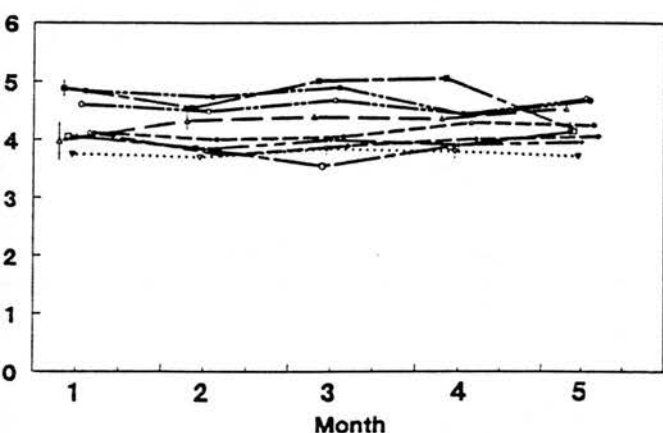
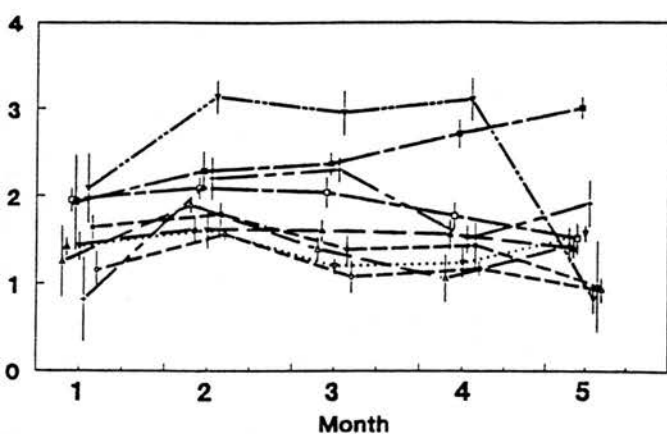


FIG 1: Long term variability of C_{qst} (litres kPa^{-1}) and $V_{A,eff}$ (litre $^{-1}$) measurements made in nine sheep at monthly intervals over a period of five months. Error bars in the upper graph represent the 95 per cent confidence limits for the slope of the regression line between pressure and volume measurements, that is, the compliance. Error bars in the lower graph represent \pm SD.

prediction intervals for individual lung function variables were calculated over the range of bodyweights studied.

Results

Long term variabilities of C_{qst} and $V_{A,eff}$ are

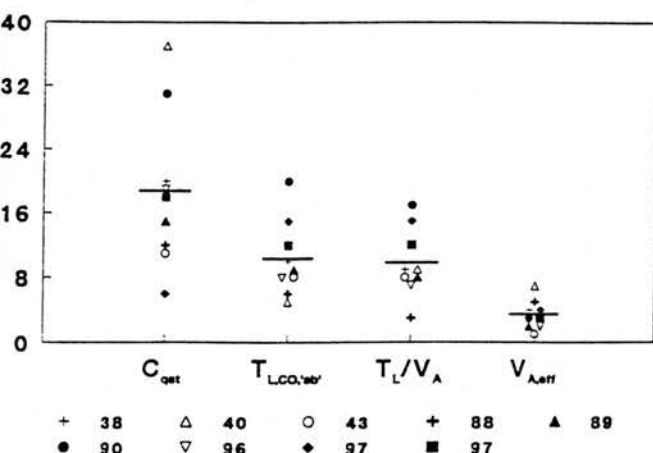


FIG 2: Individual coefficients of variation for repeated measurements of C_{qst} , $V_{A,eff}$, $T_{L,CO,'sb'}$ and T_L/V_A made in nine sheep at monthly intervals over a period of five months. The horizontal lines represent the mean values.

TABLE 1: Results of analysis of variance for repeated C_{qst} , $V_{A,eff}$, $T_{L,CO,'sb'}$ and T_L/V_A measurements made in nine sheep at monthly intervals over a period of five months

	Between days F ratio	Significance	Between sheep F ratio	Significance
C_{qst}	2.32	NS	5.77	***
$V_{A,eff}$	1.33	NS	19.94	***
$T_{L,CO,'sb'}$	2.60	NS	12.77	***
T_L/V_A	2.15	NS	11.31	***

For all variables there was no significant differences between days. Differences between sheep were highly significant. NS Not significant, $P > 0.05$; *** $P < 0.001$.

illustrated in Fig 1. Coefficients of variation for repeated measurements of each variable in individual sheep are illustrated in Fig 2. The average intrasubject CV for the repeated measurements was 18.8 ± 9.23 (mean \pm standard deviation) for C_{qst} , 10.3 ± 4.45 for $T_{L,CO,'sb'}$, 9.8 ± 4.02 for T_L/V_A and 3.4 ± 1.71 for $V_{A,eff}$. The results of the analyses of variance are shown in Table 1. There were no

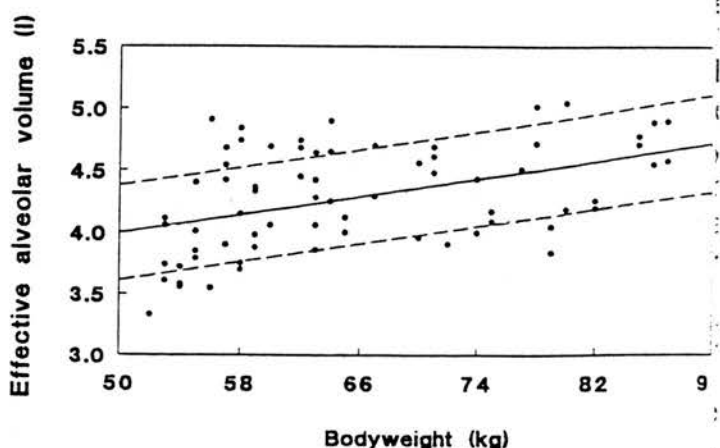
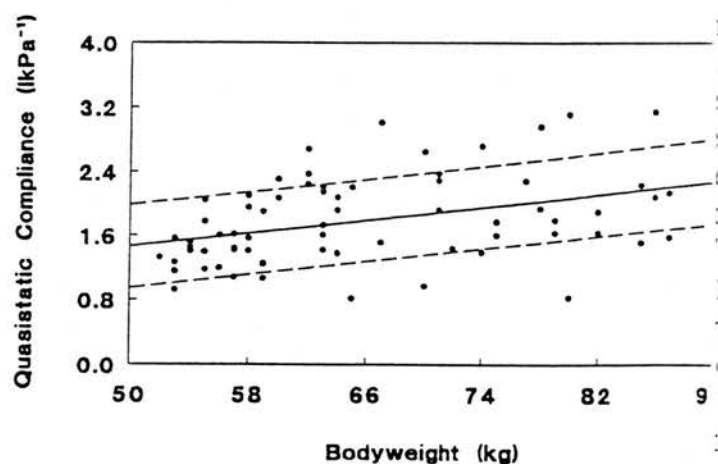


FIG 3: Relationship between C_{qst} (litres kPa^{-1}) and $V_{A,eff}$ (litre $^{-1}$) and bodyweight (kg). Repeated measurements were made in 16 sheep. Solid lines depict the regression equations that best represent the relationship between the dependent variables and bodyweight. Dotted lines represent the 95 per cent prediction intervals for the dependent variables in individual subjects.

Table 2: Selected regression equations relating C_{qst} (litres kPa^{-1}), $V_{A,eff}$ (litre $^{-1}$), $T_{L,CO_2, 'sb'}$ (mmol min^{-1} kPa^{-1}) and T_L/V_A (mmol min^{-1} kPa^{-1} litre $^{-1}$) to bodyweight (kg)

	Unit	Regression equation	F	r^2 (%)	P
C_{qst}	(litres kPa^{-1})	$0.460+0.0201 \times BW$	11.72	14.9	0.001
	(litre $^{-1}$)	$3.09+0.0181 \times BW$	17.53	20.7	<0.001
	(mmol min^{-1} kPa^{-1})	$3.27+0.110 \times BW$	40.79	37.8	<0.001
	(mmol min^{-1} kPa^{-1} litre $^{-1}$)	$1.52+0.0143 \times BW$	10.47	13.5	<0.005

ance ratio, r^2 Determination coefficient (expressed as a percentage), P Degree of significance of the variance ratio

significant differences between days for the for any of the lung function variables though between sheep differences were highly significant.

A significant relationship was found between all measured variables and bodyweight. These relationships are illustrated in Figs 3 and 4. The significant regression equations were linear variables and these equations, together with variance ratio (F), the determination coefficient (r^2) and degree of significance of the variance ratio are shown in Table 2.

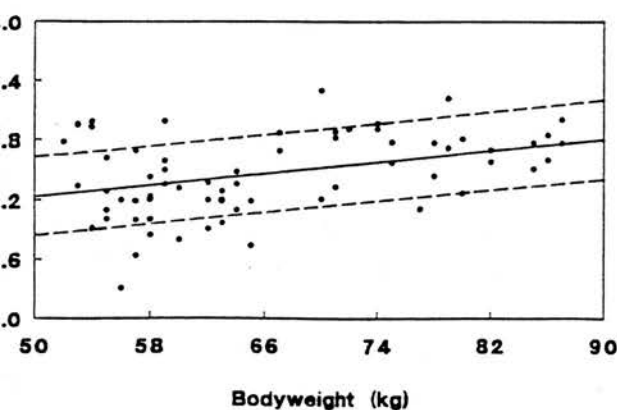
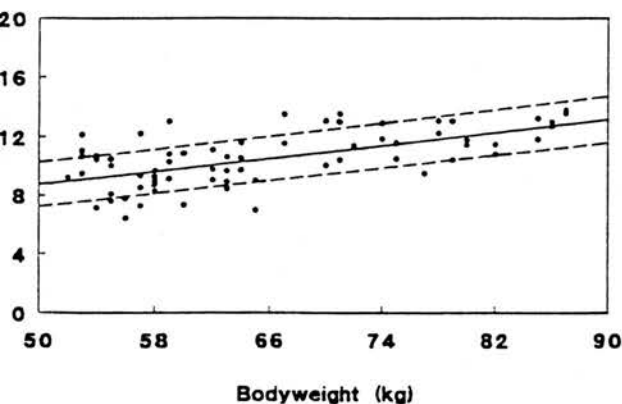


Figure 3: Relationship between $T_{L,CO_2, 'sb'}$ (mmol min^{-1} kPa^{-1}) and bodyweight (kg). Repeated measurements were made in 16 sheep. Solid lines depict the regression equations that best represent the relationship between the dependent variables and bodyweight. Dotted lines represent the 95 per cent prediction intervals for the dependent variables in individual subjects

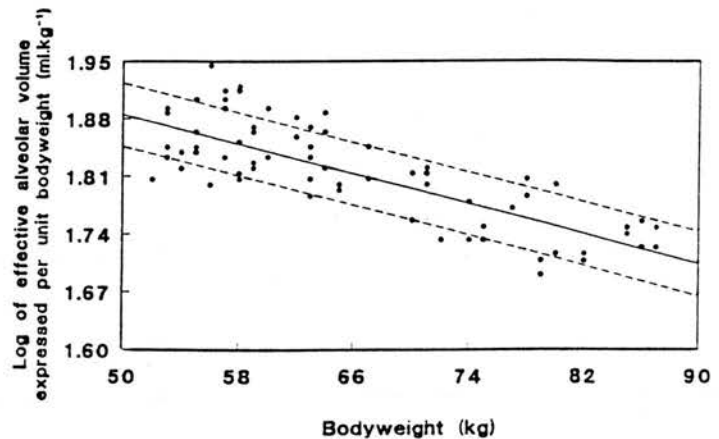


FIG 5: Relationship between $\log sV_{A,eff}$ (ml kg^{-1}) and bodyweight. Repeated measurements were made in 16 sheep. Solid lines depict the regression equation that best represents the relationship between the dependent variable and bodyweight. Dotted lines represent the 95 per cent prediction intervals for the dependent variable in individual subjects

Discussion

The predictions of Stahl (1967) encompassed the variables measured in this study and these variables were shown to be positively correlated with bodyweight. For mammals within the range of weights studied in this paper (52 to 87 kg), Stahl's (mean) predictions are 1.53 to 2.66 litre kPa^{-1} for lung compliance, 6.67 to 11.99 mmol min^{-1} kPa^{-1} for diffusing capacity, 1.876 to 1.976 mmol min^{-1} kPa^{-1} litre $^{-1}$ for diffusing capacity/TLC and 3.5 to 6.0 litres for total lung capacity.

Values of $V_{A,eff}$ and C_{qst} for the heavier bodyweight sheep were lower than Stahl's predictions and the converse was true for T_L/V_A , that is, measured values for the heavier sheep were greater than predicted. The latter finding is presumably a reflection of the reduction in $V_{A,eff}$. One explanation for these anomalies would be that rather than reflecting an increase in skeletal dimensions, greater weight in these sheep reflects an increase in adipose and perhaps muscle tissue. That this is indeed likely is shown by the reduction in specific effective alveolar volume ($sV_{A,eff}$), that is, alveolar volume expressed per unit bodyweight, with

creasing bodyweight in these sheep (Fig 5). The ratios of various lung volumes to bodyweight are relatively constant and independent of size, that is, they do not change appreciably between species (Ahl 1967). The reduction in $sV_{A,eff}$ with increasing bodyweight indicates that overfeeding sheep can deleteriously alter the normally optimal relationship between body size and lung functional capacities. This is a recognised concept in humans (Schoenberg et al 1978) where increased muscularity or obesity have opposite effects on ventilatory function. The above hypothesis does not, however, explain the agreement between measured and predicted $T_{L,CO,'sb'}$. As is thought to be the case in dogs (Robinson et al 1972), $T_{L,CO,'sb'}$ in sheep may be related to metabolic bodyweight rather than bodyweight per se.

One previous study of respiratory variables in anaesthetised sheep of bodyweight 42.6 ± 4.7 kg (mean \pm SD) reported a value of dynamic lung compliance of 1.08 ± 0.316 litre kPa^{-1} (mean \pm SD) (Halmagyi and Colebatch 1961). This low value may simply be a reflection of the method of anaesthetic maintenance they used. In their study mechanical ventilation was used during anaesthesia, and, therefore, progressive atelectasis of dependent lung units and reduced lung compliance is a likely consequence. In the present study, pentothone was administered as a single bolus dose, and so the level of anaesthesia would have varied during the procedure. As a consequence, dynamic lung volumes and mechanical and gas exchange properties of the lung would also have varied (Southorn et al 1980). This variation will necessarily be included in the measurements made, however, the adoption of a standardised dosage regime and procedural protocol will have helped to minimise such between procedure variability.

The observed regression equations relating bodyweight to lung function variables should have valuable in predicting normal values for sheep of similar breed, age, sex, weight and conformation to those used in the present study and applied under identical conditions. The 95 per cent prediction intervals were calculated using the estimated standard deviation of the dependent variables over the range of weights studied (Gardner and Altman 1989). These intervals serve to define the limits of normality for measurements of pulmonary function variables made in sheep with similar characteristics to those studied. The use of

the standard deviation as a measure of variability within a population is appropriate where the data are homoscedastic, that is, the variation is independent of the magnitude of the value measured. There are, however, inadequate data points collected in this study to determine accurately whether the data are homo- or heteroscedastic.

A knowledge of the intrasubject variation over a period of time is essential if time sequential changes in pulmonary function values for individuals are to be accurately interpreted. In human respiratory research, data are often treated as homoscedastic and reproducibility is expressed in terms of the CV. By expressing the long term variability for repeated measurements in terms of the CV, knowledge of what would constitute a significant change in an individual's lung function can be gained. Given the calculated average CVs for repeated measurements in normal anaesthetised sheep (Fig 2), it can be predicted (Pennock et al 1981) that significant ($P < 0.05$) month-to-month changes are approximately 30.8 per cent in the C_{qst} , 16.9 per cent in the $T_{L,CO,'sb'}$, 16.1 per cent in the T_L/V_A and 5.6 per cent in the $V_{A,eff}$ measurements. Previous reports have demonstrated similar intrasubject variability of lung function measurements in humans (Guyatt et al 1978), ponies (Derksen et al 1981) and cattle (Gallivan and McDonnell 1988) and illustrate the problems of interpreting lung function in individuals over time.

For all of the measured variables, the variability between repeated measurements on the separate days for the group was insignificant, whereas the variation between individual sheep was highly significant. Collective lung functional measurements on groups ($n=9$) of normal anaesthetised sheep do not therefore change significantly over time. Gallivan and McDonnell (1988) in repeated analyses on four conscious adult cows found significant differences between days for the group for respiratory frequency and inspiratory and expiratory times and concluded that a control group should be used during any long-term study on small groups of animals.

Knowledge of both the predicted normal range for the variables C_{qst} , $V_{A,eff}$, $T_{L,CO,'sb'}$ and T_L/V_A and awareness of the long term reproducibility of measurements of these variables in anaesthetised adult Texel sheep will allow more accurate interpretation, on a research basis, of respiratory disease in similar animals.

Acknowledgements

This work was supported by the Agricultural Food Research Council (grant AG15/576). The authors wish to thank Mrs A. Baker and colleagues for their care and management of the sheep during this investigation.

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Received July 7, 1992

Accepted January 21, 1993

Exponential analysis of the pressure-volume characteristics of ovine lungs

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(Accepted 4 October 1993)

Abstract. Static pressure-volume curves were generated from data obtained from 18 normal anaesthetized adult sheep. Lung volumes were determined by helium dilution. An exponential curve of the form $V = V_{\max} - Ae^{-KP}$ was fitted to the pressure-volume data from each sheep where P is the static recoil pressure. V_{\max} represents the volume asymptote, A is the difference between V_{\max} and the intercept on the volume axis and K defines the slope and hence the shape of the P-V curve. Quality of fit of the data was assessed visually, by means of a sign test and a runs test and by the coefficient of determination (r^2). Exponential equations were found to adequately describe the shape of the pressure-volume curve in sheep. The exponent K was not correlated with effective alveolar volume ($V_{A_{eff}}$) ($r_s = 0.183$; $P > 0.05$). Static lung compliance was determined over a volume range from the end-expiratory level (VEEL) to VEEL plus 400 ml. Measurements of static lung compliance were significantly correlated with measurements of effective alveolar volume ($V_{A_{eff}}$) ($r_s = 0.505$; $P < 0.025$). In the ovine, the exponent K , an index of distensibility, is independent of lung volume and offers a means of assessing lung distensibility in this species.

Distensibility, lung; Mammals, sheep; Mechanics of breathing, static pressure-volume; Pressure, static, lung volume; Volume, lung, static pressure

Static lung compliance (Cst), analysed as the lung volume change per unit pressure change (dV/dP) in the tidal volume range, is often assumed to be an index of lung elastic recoil, and is frequently used as such. Unfortunately, when comparing animals of different body size or comparing animals with varying stages of lung disease, the above assumption is subject to qualifications which limit the value of this measurement.

Firstly, variation in the end-expiratory level will alter the portion of the curve over which the compliance measurement is made *i.e.* the measurement of compliance will depend on the end-expiratory lung volume, and secondly, compliance values intuitively depend on the inflatable volume of the lungs themselves. Thus, accurate interpretation

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of compliance values demands both a knowledge of the lung volumes over which the measurement is made and a perception of the predicted normal value for the animal concerned. These latter requirements place an extra burden on measurement protocol and rely heavily on the accuracy of prediction equations based on anthropometric data.

An alternative means of analysing pressure-volume curves was pioneered by Salazar and Knowles (1964) who used an exponential equation to describe the pressure-volume curve of human adults. Glaister *et al.* (1973) employed the same principle to describe the pressure volume curves of excised dog and monkey lungs. The basis behind this method of analysis, which utilizes all the pressure and volume data points collected, has been developed and applied to the analysis of pressure-volume curves from both healthy adults (Pengelly, 1977; Colebatch *et al.*, 1979a,b; Gibson *et al.*, 1979; Knudson and Kaltenborn, 1981; Colebatch and Ng, 1986) and those with respiratory disease (Gibson *et al.*, 1979; Colebatch *et al.*, 1985; Thompson and Colebatch, 1989). The exponent K of the fitted exponential equation is not influenced by absolute values of pressure or volume and only delineates the relative changes of one with respect to the other. In other words, K is an index of pulmonary distensibility and appears not to suffer from the aforementioned limitations of compliance measurements.

Due to structural and functional similarities between the lungs of sheep and those of humans, this species has frequently been used to model human lung disease. Although pulmonary mechanics measurements are frequently included in such studies, there has, to the authors knowledge, been only one attempt to fit an exponential equation to the normal ovine pressure-volume curve (Schroter, 1980). In view of the relative advantages of expressing pulmonary distensibility in this way we sought to determine whether the ovine pressure-volume curve could be adequately described by an exponential equation and to report normal values of K for this species such that in future the distensibility of the ovine lung could be accurately assessed in disease and in relation to other species.

Materials and methods

Animals. 18 adult Texel and Texel-cross female sheep (median bodyweight 63.5; range 54–82 kg) were used in this study. The sheep were fed on a diet of proprietary concentrate and hay and were assessed to be free of significant cardiopulmonary dysfunction on the basis of a thorough clinical examination.

Anaesthesia. Sheep were starved for 12 h prior to anaesthesia which was achieved using intravenous administration of thiopentone sodium at a dose rate of $20 \text{ mg} \cdot \text{kg}^{-1}$ bodyweight. The sheep were then intubated using cuffed endotracheal tubes (diameter 9.5–10.5 mm) and placed in sternal recumbency with the head supported on a cushioned rest. A mechanical ventilator (Manley MN2; Hutchinson Blease) was adjusted to maintain a tidal volume of $10 \text{ ml} \cdot \text{kg}^{-1}$ bodyweight and respiratory rate of $10 \text{ breaths} \cdot \text{min}^{-1}$.

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Measurements. Transpulmonary pressure was measured using a differential pressure transducer (CS9; Mercury Electronics) which was connected to an oesophageal balloon catheter assembly and to a port at the proximal end of the endotracheal tube. The catheter (3 mm ID, 4.5 mm OD) had a number of holes cut in a spiral manner in the end covered by a latex balloon (length, 15 cm; diameter, 1.5 cm; thickness, 0.05 mm) and this assembly was passed into the middle thoracic portion of the oesophagus. Prior to each series of measurements, the pressure-volume characteristics of the balloon-catheter assembly were validated according to the method of Senterre and Geubelle (1970). The balloon was then evacuated and 2 ml of air was added (this volume being within the range of high compliance of this pressure recording system and close to the minimal relaxed volume of the balloon in air). The positioning of the balloon catheter assembly was confirmed by observing cardiogenic oscillations in the transpulmonary pressure recorder trace and by the absence of any deflection of this trace during inspiratory efforts against a closed airway (Milic-Emili *et al.*, 1964). Respiratory flows were measured using a Fleisch pneumotachograph (No 2) connected to a second differential pressure transducer (CS9; Mercury Electronics). Integration of the derived signal yielded respiratory volumes which were displayed on the chart recorder (Linseis L6514; Belmont Instruments). Pressure and volume recording equipment were calibrated using a water manometer and calibrated syringe respectively.

Lung volumes were measured using a single-breath helium dilution technique performed in conjunction with the measurement of single-breath transfer factor as previously described (Collie *et al.*, 1993). Lungs were inflated from the resting end expiratory level to a transpulmonary pressure of 3 kPa (defined as total lung capacity (TLC)) with a carbon monoxide and helium in air gas mixture (helium 14%, carbon monoxide 3%, balance air). After a 10-sec breathhold, a sample of alveolar gas was obtained and the concentration of helium determined (Resparameter Mk 4; P.K. Morgan). Effective alveolar lung volume (VA_{eff}) was calculated as previously described (Collie *et al.*, 1993).

Pressure-volume data were obtained after three forced inflations to TLC. The lungs were again inflated to TLC, however a stepwise deflation was achieved by intermittently interrupting the airway opening for 2–3 sec intervals.

Three collections of lung volume and pressure-volume data were made on each animal during the period of anaesthesia. To obtain absolute values for pressure-volume curves the expired volumes were subtracted from the VA_{eff} values determined immediately prior to collection of pressure-volume data. Static lung compliance (Cst) was calculated as the slope of the pressure-volume curve between the end-expiratory level and this level plus 400 ml.

Exponential analysis of pressure-volume curves. An exponential curve of the form $V = V_{max} - Ae^{-KP}$ was fitted to the data from each sheep where P is the static recoil pressure, V_{max} represents the volume asymptote, A is the difference between V_{max} and the intercept on the volume axis and K represents the slope of the P-V curve and therefore defines its shape. The method of Pengelly (1977) was used to determine the

exponential function that best fitted the data. Briefly, by expressing the difference between V_{max} and the measured volume as a fraction of V_{max} and taking the natural logarithm of this value a straight line plot with P is obtained. Least squares regression is then used to solve for slope and intercept. However, since V_{max} is unknown at the outset an iterative regression is conducted until values of the coefficient of determination (r^2) are maximized. Curves were fitted both over the whole data range and over a restricted data range in which the data points less than 50% of TLC were excluded from the analysis. In addition to assessing r^2 , curves were evaluated visually for goodness of fit and both a sign test for distribution of the data about the fitted regression line and a runs test to determine whether the order of the data was random ($P < 0.05$) were performed (Gibson *et al.*, 1979). Associations between variables were assessed using the Spearman rank correlation coefficient (r_s)

Results

The summary results of curve-fitting analysis over the whole data range for 18 sheep are shown in Table 1. The pressure-volume data from two sheep with exponential curves fitted are presented in Fig. 1. These examples represent the opposite ends of the spectrum in terms of goodness of fit as assessed by r^2 . In general the regression lines appeared to closely fit the pressure-volume data on visual inspection. Although only one pressure-volume curve failed the sign test ($P < 0.05$), all but two failed the runs test ($P < 0.05$) indicating that there were systematic deviations from the fitted regression lines in almost all the sheep *i.e.* the order of these deviations was not random. The nature of these deviations is demonstrated in a plot of the residuals against pressure (Fig. 2) (with pressures expressed as percentages of the range between the minimum (0%) and maximum (100%) pressure recorded for each set of data points). The most obvious systematic deviations from the fitted regression lines occurred when transpulmonary pressure was maximal with measured volumes being greater than the

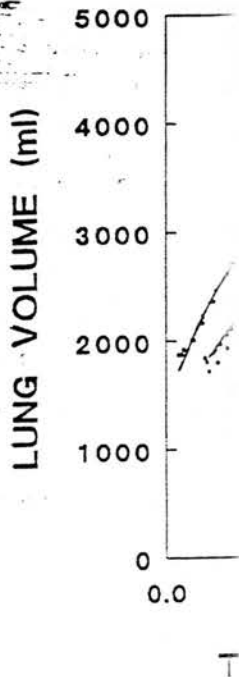


Fig. 1. Pressure-volume data for two sheep.

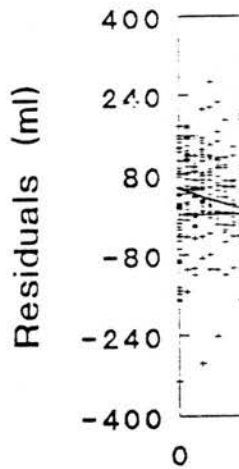


Fig. 2. Plot of residuals for pressure-volume data for two sheep.

TABLE 1

Summary results from exponential pressure-volume curve fitting analysis of the form $V = V_{max} - Ae^{-KP}$ in 18 normal sheep. V_{max} is the volume asymptote. A is the difference between V_{max} and the intercept on the volume axis. K defines the slope and hence the shape of the fitted pressure-volume curve and r^2 is the coefficient of determination of the regression. Cst is the static lung compliance.

Measurement	Median	Range
Data points animal	41	26-48
K (kPa^{-1})	1.456	1.091-2.119
A	2542	1590-3252
V_{max} (ml)	4168	3277-4896
r^2	98.74	96.11-99.74
Cst ($l.kPa^{-1}$)	2.050	1.131-3.698

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Range
26-48
1.091-2.119
1590-3252
3277-4896
96.11-99.74
1.131-3.698

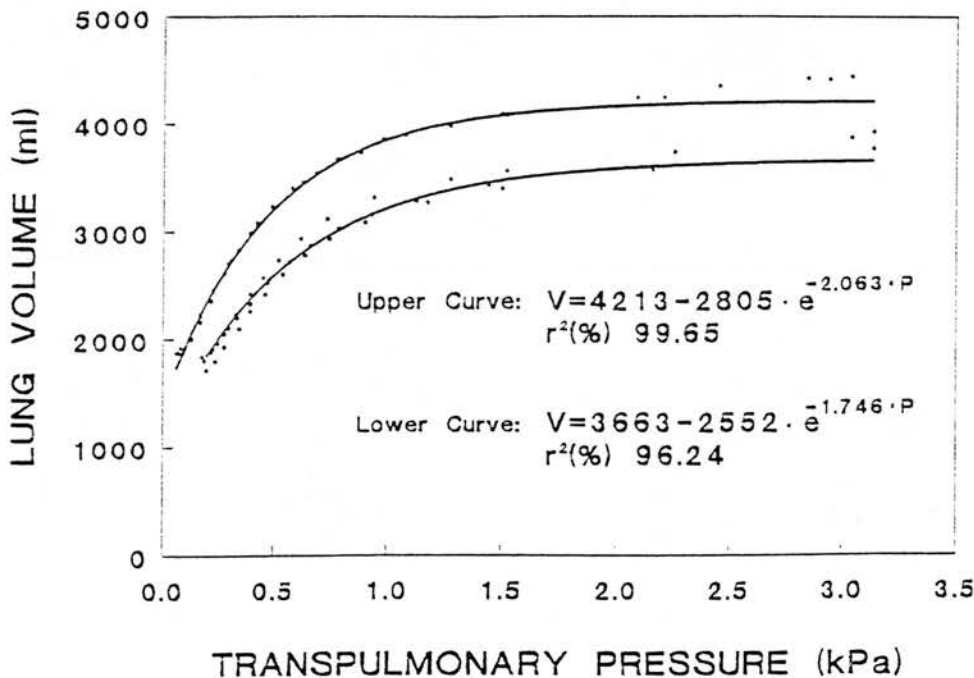


Fig. 1. Pressure-volume curves from two normal sheep. The two curves are representative of the extremes of quality of fit of data.

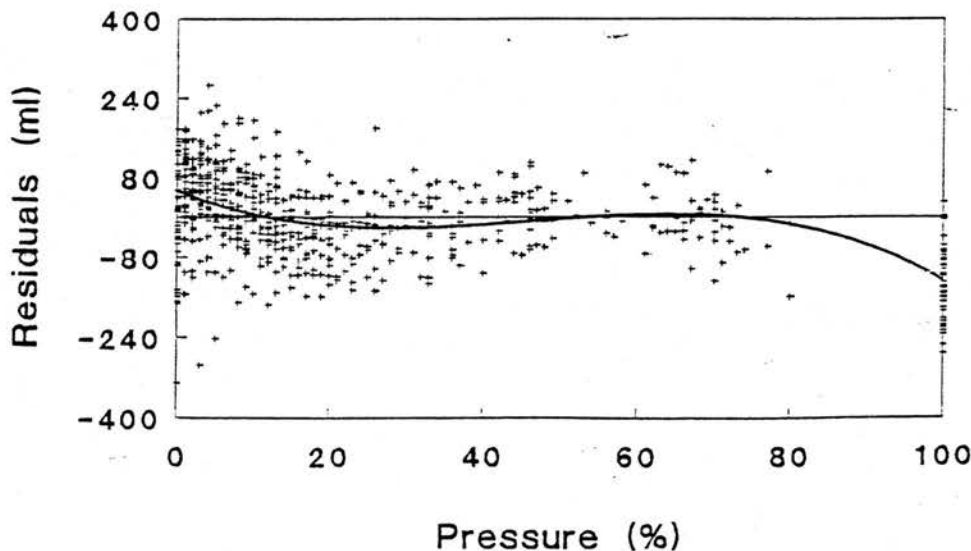


Fig. 2. Plot of residuals for all fitted curves for 18 sheep. Pressures are expressed as percentages of the range between the minimum (0%) and maximum (100%) pressure recorded for each set of data points. The fitted curve is of the form $Y = a + b \cdot x + c \cdot x^2 + d \cdot x^3$.

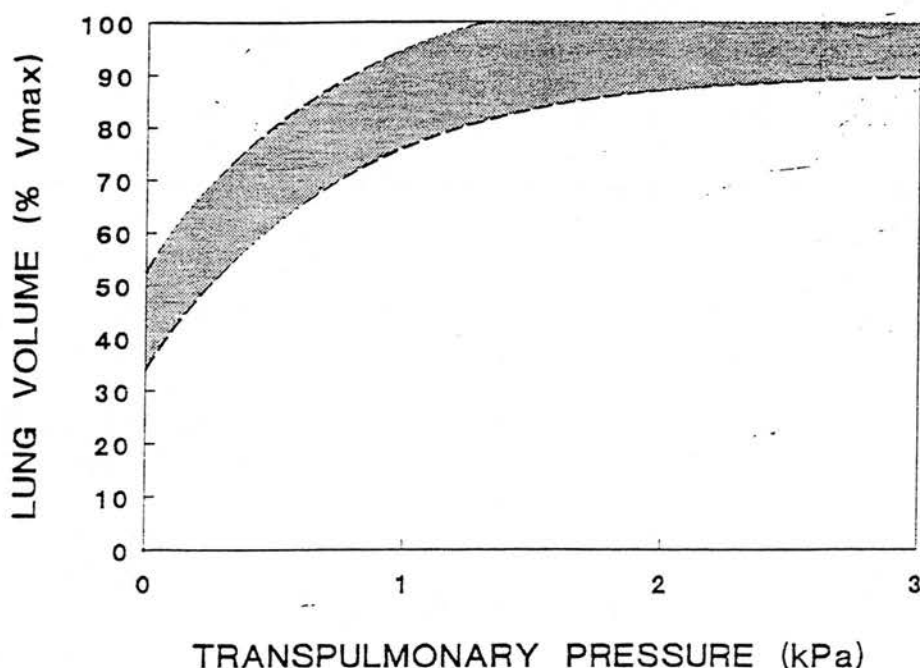


Fig. 3. Relationship between transpulmonary pressure and lung volume (expressed as percentage of predicted V_{max}) in normal sheep. The shaded area represents the 95% confidence interval.

regression would seem to predict. Curves fitted data well at lower volume limits. Curve fitting over the restricted data range improved the quality of fit (as assessed by r^2) in only one instance. In the remaining sheep, fitting a curve to the restricted data range led to a slight reduction in r^2 (median 98.34; range 95.42–99.39) and K (median 1.348; range 0.820–2.098), and an increase in V_{max} (median 4202; range 3468–4896).

The iterative regression was repeated on the pooled data from all sheep with volume expressed as percent of V_{max} . The exponential equation $V/V_{max} (\%) = 100 - 56.7 \cdot e^{-1.37 \cdot P}$ was found to best fit this pooled data. The relationship between transpulmonary pressure and lung volume expressed as percent predicted V_{max} is depicted in Fig. 3 with the shaded area representing the 95% confidence interval for normal sheep.

A significant positive correlation was apparent between C_{st} and VA_{eff} ($r_s = 0.505$; $P < 0.025$) whereas no significant relationship existed between K and VA_{eff} ($r_s = 0.183$; $P > 0.05$).

Discussion

The best-fit exponential curve was chosen by maximizing the coefficient of determination obtained by least-squares regression analysis of the linearised data. Visually, exponential curves appeared to closely fit the pressure-volume data and this was reflected

in the regression variable was explained. Variation exceeding 2

Systematic deviation of transpulmonary pressure from the portion of the curve at low volumes measured by an exponential curve. Transpulmonary pressure published pressure-volume data (Halmagyi, 1961) support the second assumption could be derived to a hyperbolic-sigmoidal volume data than relative simplicity of predictable effect of weight heavily in its of the curve at high potential constant pressure-volume analysis, whereas only data in the calculation of is independent of time in comparative static mechanics.

Data points at low volumes in humans (Colebatch, 1971) and healthy men (Sutheby, 1971) by the sequential data points below this point to underestimation of points were not well to underestimate V_{max} below 50% TLC of the factors responsible in anaesthetized sheep can be used for extrapolation.

The results of this study are approximately 57%. This primary pressure axis is described for human (Kaltenborn, 1981).

in the regression analyses, where 96.11–99.74% of the variation in the dependent variable was explained by the fitted curve. Indeed, in only 4 cases did the unexplained variation exceed 2% of the total variation.

Systematic deviations from the exponential functions were frequently noted at high transpulmonary pressures and much of the error variance was attributable to this portion of the curve. The implications of these observations are that (a) in sheep, lung volumes measured at a transpulmonary pressure of 3 kPa are not maximal, and (b) an exponential curve is not the most accurate description of the relationship between transpulmonary pressure and lung volume in this species. Examination of a previously published pressure-volume curve obtained from an anaesthetized sheep (Colebatch and Halmagyi, 1961) suggests that the first assumption might be valid. With regard to the second assumption, it is quite likely that a more complex mathematical formulation could be derived to better fit the pressure-volume data from sheep, just as in humans a hyperbolic-sigmoid model appears to offer a better fit to the full range of pressure-volume data than the exponential model (Murphy and Engel, 1978). However, the relative simplicity and lack of constants in the exponential equation together with the predictable effect of a change in any constant on the shape and position of the curve weigh heavily in its favour and, in our opinion, compensate for the poor description of the curve at high transpulmonary pressure. Certainly, it would appear that the exponential constant K holds several advantages over C_{st} measurements in describing pressure-volume curves. Firstly, the full range of data is used in the exponential analysis, whereas only data points in the linear portion of the pressure-volume curve are used in the calculation of C_{st} . Secondly, whereas C_{st} is positively correlated with VA_{eff} , K is independent of this variable and thus is a more useful index of distensibility for use in comparative studies or when studying the effects of lung disease on pulmonary mechanics.

Data points at low volume levels tend not to conform to the exponential model in humans (Colebatch *et al.*, 1979a; Gibson *et al.*, 1979). Below about 46% TLC in seated healthy men (Sutherland *et al.*, 1968) there is an inflection in the curve caused primarily by the sequential closure of small airways and oesophageal artefacts. Inclusion of data points below this point of airway closure in the exponential curve fitting analysis leads to underestimation of K and overestimation of V_{max} (Gibson *et al.*, 1979). Inflection points were not well defined in our ovine pressure-volume curves and curves appeared to underestimate V_{max} rather than the converse. In addition, exclusion of data points below 50% TLC did not improve the quality of curve fit. We therefore conclude that the factors responsible for curve inflection have less influence in the low volume range in anaesthetized sheep relative to humans and the whole data range from VEEL to TLC can be used for exponential curve fitting.

The results of the pooled regression indicate that the ratio of $A/V_{max}\%$ is approximately 57%. This ratio describes the position of the curve relative to the transpulmonary pressure axis and is considerably lower than the equivalent ratio previously described for humans (Colebatch *et al.*, 1979a; Gibson *et al.* 1979; Knudson and Kaltenborn, 1981) indicating that relative to these studies, the sheep pressure-volume

curves are displaced to the left. Knudson and Kaltenborn (1981) reason that differences in oesophageal balloon volume can account for much of the variation in this ratio seen in human studies and comment that, compared to measurements of K which are seemingly less variable between studies, the value of this ratio to compare various studies is limited.

K does not seem to be volume dependent in humans (Colebatch *et al.*, 1979a) although a progressive increase in this parameter occurs with age (Colebatch *et al.*, 1979b) such that between 20 and 80 years of age the value of K would be expected to increase from approximately 1.224–1.748 kPa. The age related increase in K is believed to be due to increased unstressed alveolar dimensions and reduced total surface acting forces (Colebatch and Ng, 1986). This relationship between airspace size and K has been demonstrated in excised human lungs (Greaves and Colebatch, 1980) and for lungs of several mammalian species (Haber *et al.*, 1983). Thus, species with very small alveoli would be expected to have relatively large surface acting forces and low values of K . In this regard it is interesting that the values of K we have obtained for adult sheep (median 1.456 kPa; range 1.091–2.119) are similar to those reported for humans; this despite sheep having smaller alveolar dimensions and correspondingly greater alveolar surface density relative to humans (Gehr *et al.*, 1978; Warner *et al.*, 1986). Whether this represents an intrinsic difference between species with regard to the type and extent of tissue related forces or a difference due to methodological inconsistencies between studies remains to be ascertained.

In conclusion, our studies indicate that an exponential equation adequately describes the whole pressure-volume curve of normal anaesthetized sheep. The index of distensibility K , is independent of lung volume and offers a means of comparing lung distensibility both with other species and during lung disease in this species.

Acknowledgements. The authors are indebted to Alison Baker for her technical assistance. This study was supported by Agriculture and Food Research Council grant AG15/576.

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